Characterization of *Arabidopsis* WYR and its Potential Role in Non-Random Sister Chromatid Segregation

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Roger Schmid

von

Seon AG

Promotionskommission

Prof. Dr. Ueli Grossniklaus (Vorsitz)

Prof. Dr. Enrico Martinoia

Dr. Amar J. S. Klar

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Characterization of *Arabidopsis* WYR and its Potential Role in Non-Random Sister Chromatid Segregation

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by

Roger Schmid

Examination Committee

Prof. Dr. Ueli Grossniklaus, University of Zürich (Chairman)

Prof. Dr. Enrico Martinoia, University of Zürich

Dr. Amar J. S. Klar, National Cancer Institute, Frederick, USA

Faculty of Science, University of Zürich

Zürich, 2017

Personal Data

Curriculum Vitae

Roger Schmid

Niederlenzer Kirchweg 5c
5600 Lenzburg
Tel.: 0041 78 814 49 56
Mail: 2roger.schmid@gmail.com

Date of birth: July 21, 1986
Nationality: Swiss
Marital status: Married (1 child)

Education

February 2013 – Autumn 2016	Ph.D. in Science and Policy, University of Zürich with Ueli Grossniklaus
August 2011 – January 2013	Master of Science in Plant Science, Fast-Track, University of Zürich
September 2009 – February 2011	Master of Science in Molecular Technologies, University of Applied Sciences Northwestern Switzerland with Susan Gasser
November 2006 – July 2009	Bachelor of Science in Molecular Life Science, University of Applied Sciences Northwestern Switzerland
August 2003 – August 2006	Professional Baccalaureate (aprentas)
August 2003 – August 2006	Apprenticeship as a Chemical Lab Assistant for Novartis

Professional Activity

March 2011 – July 2011	Employment as a Lab Assistant for Biolytix AG
August 2003 – August 2006	Apprenticeship as a Chemical Lab Assistant for Novartis

Honors and Awards

September 2009	Advancement studentship „Hirschmann-Stipendium“ for Master-students with an above average Bachelor degree

Publications

Schmid R. and Grossniklaus U. in preparation: Coordinated sister chromatid segregation in *Arabidopsis thaliana*.

Schmid R. and Grossniklaus U. in preparation: *WYR*: From Asymmetry to Haploinsufficiency.

Horigome C., Oma Y., Konishi T., **Schmid R**., Marcomini I., Hauer M. H., Dion V., Harata M., Gasser S. M. (2014) SWR1 and INO80 chromatin remodelers contribute to DNA double-strand break perinuclear anchorage site choice. Mol. Cell 55, 626–639

Neumann FR, Dion V, Gehlen LR, Tsai-Pflugfelder M, **Schmid R**, Taddei A, Gasser SM. 2012. Targeted INO80 enhances subnuclear chromatin movement and ectopic homologous recombination. Genes Dev 26: 369–383.

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Summary

During the synthesis phase of the cell cycle both DNA strands of a chromatid serve as a template for the synthesis of two new DNA strands. To prevent stem cells from experiencing replication errors, the immortal strand hypothesis (ISH)[1] postulates the directed and coordinated segregation of all replication-error-free template strands into the daughter cell, which retains the stem cell's fate during consecutive asymmetric stem cell divisions. Alternatively, non-random sister chromatid segregation could have evolved for epigenetic reasons[2,3]. If two sister chromatids differ in their epigenetic marks, non-random sister chromatid segregation could affect cell fates. Plants bearing a mutation in *WYR*, the ortholog of human *INCENP*, show defects in cell fate decision during female gametophyte development[4]. Based on the function of *WYR*'s orthologs in chromosome segregation and its necessity for correct cell fate decisions[5,6], we hypothesized that *WYR* plays a role in non-random sister chromatid segregation. The aim of my doctoral project was the further characterization of *WYR* and to test for the existence of non-random sister chromatid segregation in plants. To achieve this, I developed a new method for strand-specific single cell whole genome sequencing of cells isolated from multicellular tissues. This method allowed me to track sister chromatid segregation during pollen development.

I observed the non-directed but coordinated segregation of DNA strands during pollen development. This finding of coordinated sister chromatid segregation supports the ISH. However, the absence of directed sister chromatid segregation in pollen argues against the applicability of the ISH to male gametophyte development. *wyr-1* turns out to mainly cause chromosome segregation defects during pollen meiosis, and due to the absence of directed sister chromatid segregation, plays no role in non-random sister chromatid segregation during asymmetric pollen mitosis 1.

1. Cairns, J. Mutation selection and the natural history of cancer. *Nature* **255,** cp1- (1975).
2. Lansdorp, P. M. Immortal Strands? Give Me a Break. *Cell* **129,** 1244–1247 (2007).
3. Klar, A. J. S. A model for specification of the left-right axis in vertebrates. *Trends in Genetics* **10,** 392–396 (1994).
4. Kirioukhova, O. *et al.* Female gametophytic cell specification and seed development require the function of the putative Arabidopsis INCENP ortholog WYRD. *Development* **138,** 3409–3420 (2011).
5. Cutts, S. M. *et al.* Defective chromosome segregation, microtubule bundling and nuclear bridging in inner centromere protein gene (Incenp)-disrupted mice. *Hum. Mol. Genet.* **8,** 1145–1155 (1999).
6. Resnick, T. D. *et al.* Mutations in the chromosomal passenger complex and the condensin complex differentially affect synaptonemal complex disassembly and metaphase I configuration in drosophila female meiosis. *Genetics* **181,** 875–887 (2009).

Zusammenfassung

Während der Synthesephase des Zellzyklus dient jeder DNA-Strang als Vorlage für die Synthese eines neuen DNA-Strangs. Zum Schutz der Stammzellen vor Kopierfehlern, postuliert die Hypothese unsterblicher DNA-Stränge[1] die gerichtete und koordinierte Segregation aller kopierfehlerfreien DNA-Vorlagestränge in die den Stammzellcharakter beibehaltende Tochterzelle während asymmetrischer Stammzellteilungen. Die gerichtete und koordinierte Segregation von Schwesterchromatiden könnte auch epigenetisch Sinn machen: Wenn sich zwei Schwesterchromatiden epigenetisch unterscheiden, könnte die Schwesterchromatidensegregation das Zellschicksal der Tochterzellen beeinflussen[2,3]. *WYR* ist das orthologe Gen vom menschlichen *INCENP*, welches für die korrekte Chromosomensegregation essentiell ist[4,5]. Pflanzen mit einer Mutation in *WYR* zeigen Defekte in der Festlegung des Zellschicksals während der Entwicklung des weiblichen Gametophyten[6]. Daraus leiteten wir die Hypothese ab, dass WYR eine Rolle in der Schwesterchromatidensegregation spielen könnte. Ziel meiner Doktorarbeit war die weitere Charakterisierung von WYR und das Überprüfen der Existenz gerichteter Schwesterchromatidensegregation in Pflanzen. Dazu habe ich eine neue Methode zur strangspezifischen genomweiten Sequenzierung einzelner Zellen aus Geweben entwickelt, und zeige die Existenz von ungerichteter aber koordinierter Segregation von DNA-Strängen während der Entwicklung von Pollen. Meine Entdeckung von koordinierter Schwesterchromatidensegregation unterstützt die Hypothese unsterblicher DNA-Stränge. Wegen der Abwesenheit gerichteter Schwesterchromatidensegregation kann die Hypothese jedoch nicht zur Beschreibung der Pollenentwicklung angewandt werden. Die beschriebenen Phänotypen von *wyr-1* lassen sich durch Chromosomensegregationsprobleme während der Pollenmeiose erklären.

1. Cairns, J. Mutation selection and the natural history of cancer. *Nature* **255,** cp1- (1975).
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Chapter 1 – Introduction 1

Non-random sister chromatid segregation during gametogenesis

Sexual reproduction requires haploid gametes, which fuse with each other during fertilization to form a diploid zygote. Gametes are haploid; contain only half the number of each chromosome and half the amount of genomic DNA compared to somatic cells. In contrast, zygotes are diploid and consist of the fused chromosome sets of both haploid gametes. This process is universal for sexual reproduction to prevent the duplication of the chromosome set in each generation. However, gametogenesis, the process by which precursor cells differentiate to form mature haploid gametes, differs from animals to plants. In animals (Figure 1A) gametogenesis occurs by meiotic division of diploid gametocytes into gametes whereas in plants (Figure 1B) gametogenesis occurs by mitotic division of haploid gametogenous cells. Gametogenous cells are produced from haploid spores after sporic meiosis and form the gametophyte, a multicellular haploid phase in the life cycle between meiosis and gametogenesis. Gametophytes consist of haploid cells with different cell fates of which one is the gamete cell fate. Despite the discovery of several mutants with gametophytic cell fate differentiation problems, the mechanisms for cell fate differentiation in both the male and the female gametophytes remain largely unknown. Most likely an interplay of different mechanisms is required for correct cell fate decisions. Polar expression of genes in the embryosac, the female gametophyte of seed plants, for example *DEMETER*[1], suggests a role for positional cues in gametic cell specification. A lateral inhibition mechanism is assumed for the differentiation of egg and synergid cell fates[2]. Here we suggest an additional potential mechanism that might contribute to cell fate differentiation in plant gametophytes, the non-random segregation of sister chromatids during cell divisions.

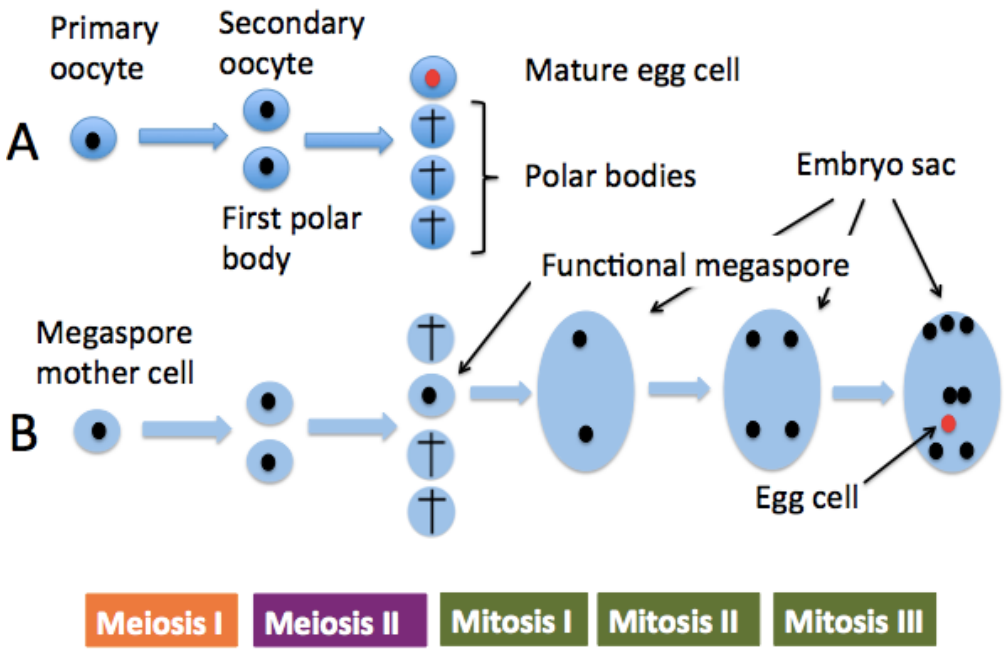

Figure 1 **A)** Animal gametes (red nucleus) are direct products of meiosis. **B)** Plant gametes differentiate not directly after or during meiosis but only after some more mitotic divisions. *Arabidopsis thaliana* female gametophyte development requires 3 post-meiotic divisions to form 7 cells consisting of 4 different cell types. Collectively they form the female gametophyte, the embryo sac.

The "somatic strand-specific imprinting and selective sister chromatid segregation" (SSIS) and the "immortal DNA strand" (ISH) hypotheses

Cell division is the process by which a parent cell divides into two daughter cells. It occurs as part of a larger cell cycle, which usually involves processes like cell growth or DNA replication and chromatid segregation. The synthesis of two new DNA strands based on the template strands before cell division leads to two new double stranded DNA chromatids, each containing a former template strand and a newly synthesized strand (Figure 2). During mitosis, the two chromatids finally segregate to both daughter cells.

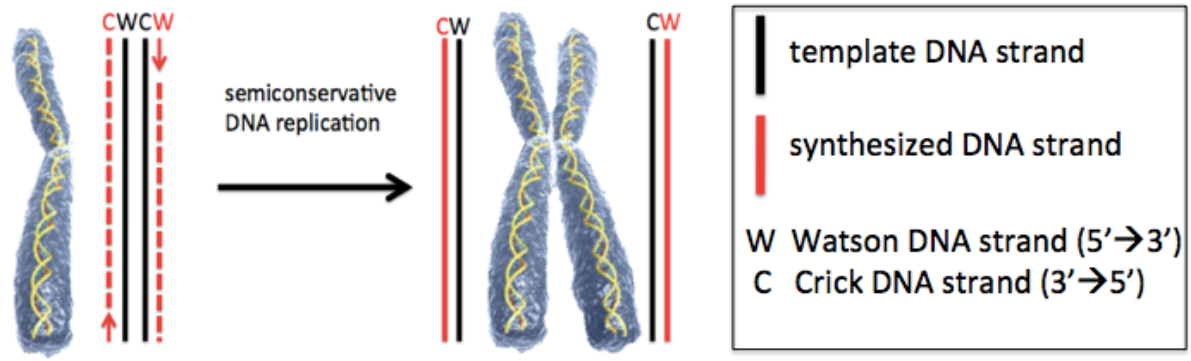

Figure 2 Semiconservative DNA replication. DNA replication synthesizes two new Watson and Crick DNA strands (red) based on the two template (black) Watson and Crick DNA strands. This process leads to two new chromatids, each consisting of a newly synthesized strand and an "old" template strand.

Semiconservative replication and mitotic segregation confer the chromatids a history. For instance, if a parent cell with two template strands goes through one round of cell division, both daughter cells end up with one chromatid consisting of one template DNA strand and one newly synthesized DNA strand (Figure 3). However, after one more round of cell division, the four daughter cells' chromatids differ in their age and acquired different histories (Figure 3). Two of the four chromatids consist of two newly synthesized strands. One DNA strand was synthesized before the first cell division and the other DNA strand before the second cell division. In contrast, the other two chromatids consist of one template strand, which was already present before the first cell division, and a DNA strand synthesized before the second cell division.

To our knowledge, it's unknown whether these historically different chromatids segregate randomly during mitosis or whether a potential mechanism specifically regulates the segregation of chromatids depending on their history.

1966-1967 Karl Lark published first results pointing toward non-random sister chromatid segregation in mammalian cells and plant root cells[3,4]. Amar Klar proposed 1994 the somatic strand-specific imprinting and selective sister chromatid segregation (SSIS) hypothesis[5] that includes the specific segregation of sister chromatids, which

differ in their epigenetic landscape (Figure 3). The specific segregation of epigenetically different chromatids is thought to influence the expression of genes and hence the cell fate of target cells. An alternative hypothesis, the immortal DNA strand hypothesis (ISH) by Cairns 1975[6], postulates that stem cells protect themselves from replication errors by the coordinated and directed segregation of error-free DNA template strands (Figure 4).

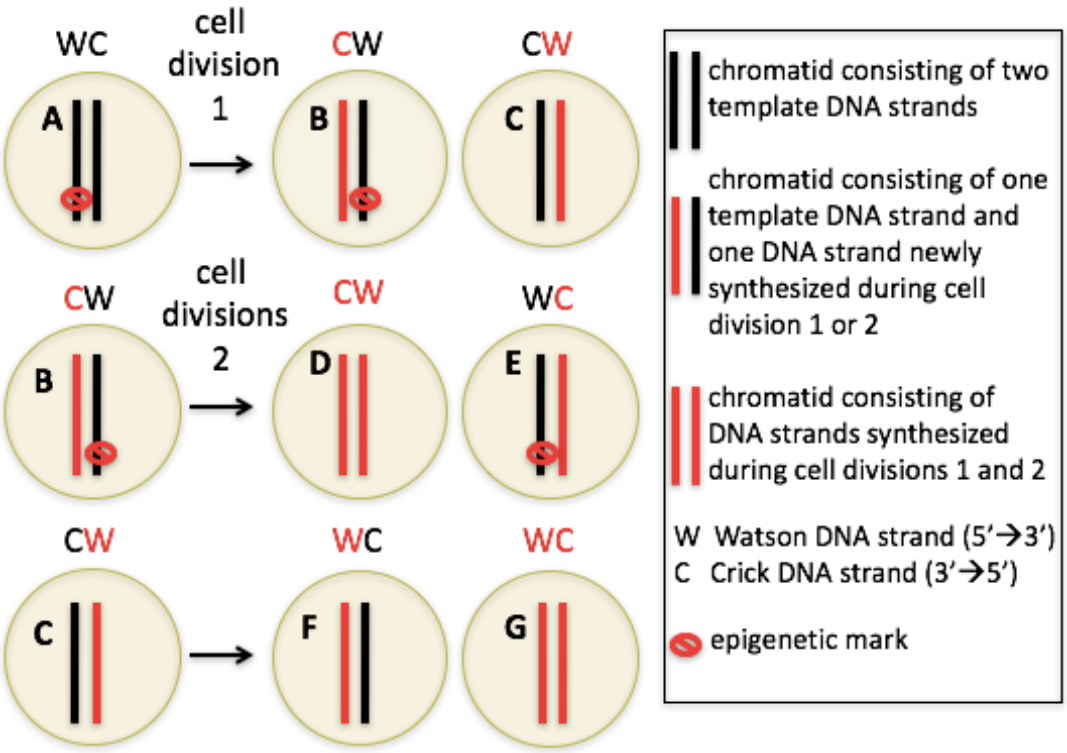

Figure 3 Strand-specific imprinting and selective sister chromatid segregation hypothesis. Parent cell A with a chromatid consisting of two template (black) DNA strands divides into two daughter cells each inheriting a former template DNA strand and a newly synthesized (red) DNA strand. The content of newly synthesized DNA versus template DNA is the same for both cells B and C. The only difference is that cell B inherited the Watson template strand whereas cell C inherited the Crick template strand. If either the "old" Watson or Crick strand bore individual epigenetic marks, which are not copied during DNA replication, cells B and C would bear different epigenetic marks and the regulated or non-random segregation of sister chromatids might influence the cell fate of both daughter cells B and C. If cells B and C go through a second cell division they divide into daughter cells with a historically different DNA content. Cell B divides into cells D and E. Cell D consists of one DNA strand synthesized before cell division 1 and one DNA strand synthesized before cell division 2. In contrast, cell E still contains one "old" template DNA strand and one DNA strand synthesized before cell division 2. Cell C divides into cells F and G. They equal cells E and D but inherited the opposite (Watson versus Crick) DNA template strands.

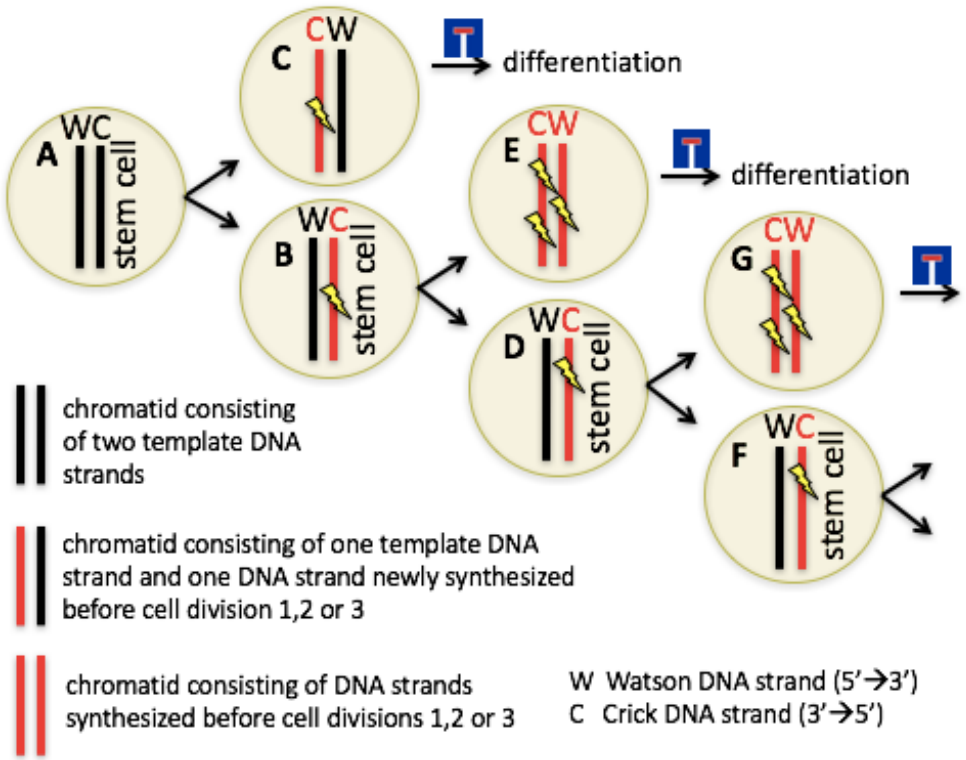

Figure 4 Immortal DNA strand hypothesis. A parent stem cell (A) with a chromatid consisting of two template (black) DNA strands divides into two daughter cells, each inheriting a former template DNA strand free of copy errors and a newly synthesized (red) copy error-containing DNA strand. One of both daughter cells (here C) differentiates and will stop dividing at some point. The other daughter cell (here B) keeps its stem cell fate and undergoes a further asymmetric stem cell division to form daughter cells D and E. However, only one of both daughter cells (here D) inherits the error-free template strand from the previous cell division. The non-random segregation of the "old" DNA template strand always to the stem cell fate-retaining cell during sequential asymmetric stem cell divisions might prevent the spread of mutations (yellow thunderbolt) caused by DNA replication mistakes.

Epigenetically divergent sisters

Gene expression varies between different cell types. Heritable changes in gene expression are studied in the field of Epigenetics and are mainly explained by epigenetic markers; heritable chemical or structural modifications of the chromatin but not of the DNA sequence.

The SSIS hypothesis depends on epigenetic differences between sister chromatids. Nevertheless, it remains largely unknown whether such differences exist. Chromatin is a complex of DNA, proteins, and RNA. DNA and DNA CG methylation marks undergo

semiconservative replication in plants and animals. Plant CHG methylation marks but most likely not CHH methylation marks undergo semiconservative replication as well[7]. In contrast, no mechanism for semiconservative replication of the chromatin protein modifications is known[8] and thus chromatin proteins are potential carriers of epigenetic differences between sister chromatids.

DNA replication requires that enzymes can access DNA and therefore most of the chromatin proteins have to be evicted from DNA before DNA replication initiates. After DNA replication the proteins reintegrate into both daughter DNA strands (Figure 5). Duplication of the chromatin during DNA replication requires chromatin proteins to be newly synthesized. Chromatin proteins acquire many different transient marks during their lifetime. During replication, older chromatin proteins are diluted with newly synthesized unmarked proteins and redistributed onto both chromatids. It remains largely unknown how old and newly synthesized proteins with different marks distribute to both sister chromatids and how marks are copied onto newly synthesized proteins. The emerging model claims that epigenetic marks are not entirely restored during cell division and that passing through chromatin replication could introduce changes in gene expression and hence differentiation[9].

Not only DNA strands have a history but also chromatin proteins need to be newly synthesized in each cell division. Age-dependent chromatin protein marks on both template DNA strands might lead to a functional difference between both daughter chromatids after cell division. The non-random segregation of these functionally different chromatids might result in different cell fates of both daughter cells.

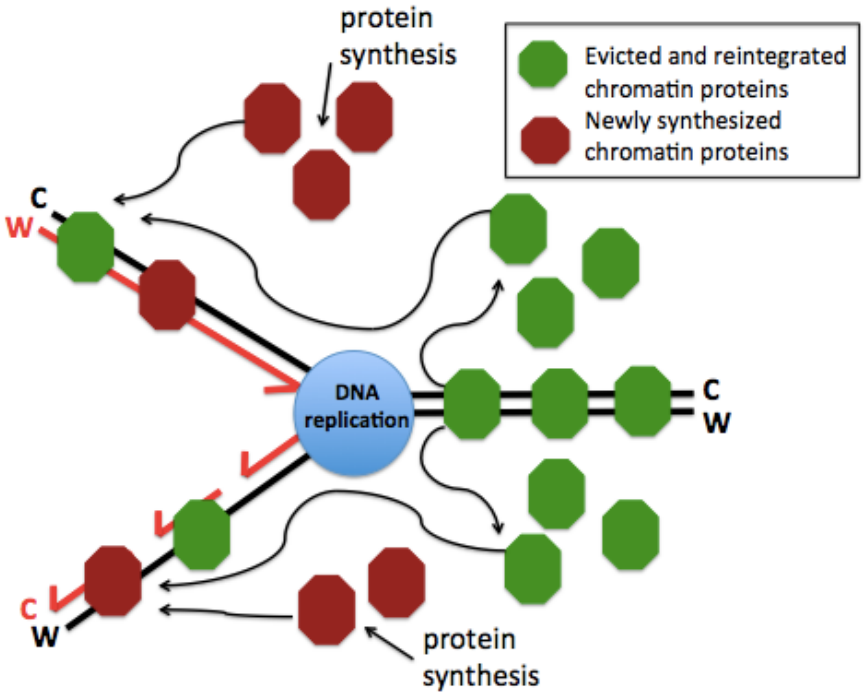

Figure 5 Chromatin replication. DNA replication only works if DNA replication enzymes can access DNA, which requires that most chromatin proteins are evicted from DNA before DNA synthesis initiates. Because chromatin replication produces two chromatids from one chromatid, new chromatin proteins (dark red) need to be synthesized. The eviction of chromatin proteins that gained several epigenetic marks during their lifetime (green) and the synthesis of unmarked new proteins (dark red) produces two sorts of chromatin proteins. How and in which ratio both types are integrated into both newly synthesized chromatids is widely unknown.

Sister chromatid segregation during plant gametophyte development

We propose the non-random segregation of functionally different chromatids as a possible mechanism for cell fate control in the plant gametophytes. Together with positional and lateral inhibition mechanisms, non-random segregation of functionally different chromatids might contribute to gamete differentiation.

The male gametophyte of *Arabidopsis thaliana* is a three-celled pollen. Its development starts from a microsporocyte, which undergoes meiosis and divides into four haploid microspores. Subsequently, all microspores undergo a highly asymmetric division, called pollen mitosis 1 (PMI) to produce four bicellular pollen grains with a small germ cell within the cytoplasm of a large vegetative cell. Finally, the germ cell undergoes one further mitotic division at pollen mitosis 2 (PMII) to form two sperm cells

(Figure 6). We suggest that the non-random segregation of functionally different chromatids in pollen mitosis 1 might contribute to the germ and vegetative cell fates (Figure 7).

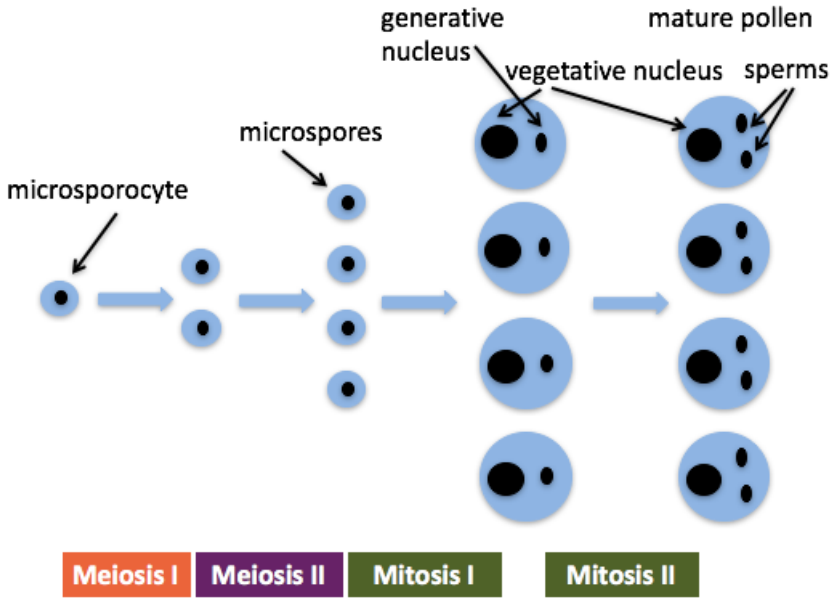

Figure 6 Pollen development in *Arabidopsis thaliana* starts with a diploid microspore that undergoes meiosis to divide into 4 haploid microspores. Each microspore enters mitosis 1 to divide into a vegetative and a generative cell. The generative cell divides once more during mitosis 2 to produce two sperms. One of both sperms will fuse with the egg cell after pollination and during fertilization. Mitosis 1 is asymmetric and forms two daughter cells of different cell types.

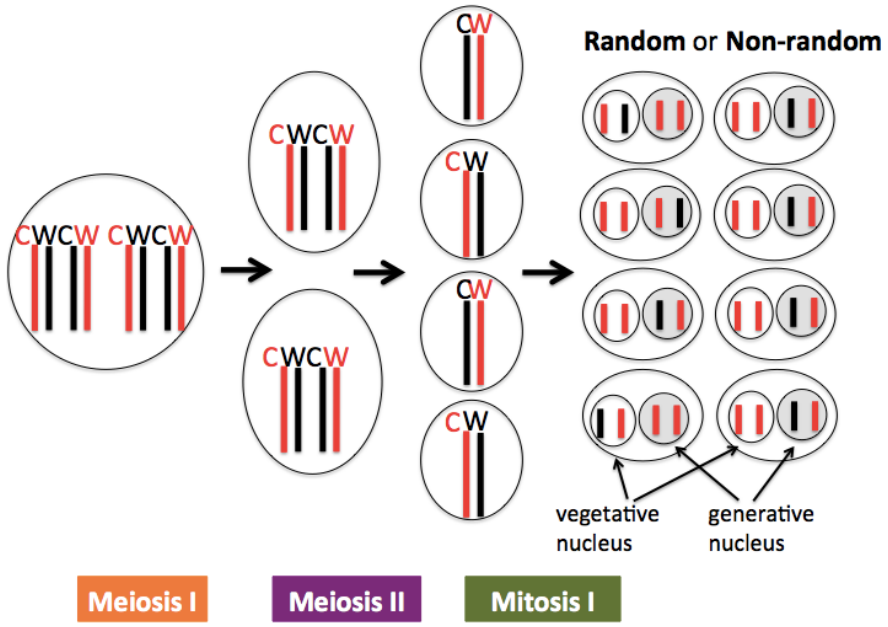

Figure 7 (Non-)random sister chromatid segregation during pollen development. Pollen development in *Arabidopsis thaliana* starts with a diploid microsporocyte that undergoes meiosis to divide into 4 haploid microspores. Meiosis produces 4 microspores each containing a template (black) and a newly (red) synthesized DNA strand. During mitosis 1, the template strand is diluted with newly synthesized strands

and only one of both produced nuclei inherits the "old" template strand (black). We propose that the segregation of the "old" template strand (black) is non-random and directed to the same cell-type, either the vegetative or generative cell.

The female gametophyte of *Arabidopsis thaliana* is a seven-celled eight-nuclear megagametophyte of the Polygonum type. Its development starts from a megaspore mother cell (MMC), which undergoes meiosis to divide into four haploid megaspores. Three of the megaspores degenerate, and just one becomes a functional megaspore (FM). Subsequently, three following mitotic divisions of the FM lead to the formation of the mature embryo sac with 7 cells of 4 different cell types (Figure 1B). We suggest that the non-random segregation of functionally different chromatids in all 3 mitotic divisions might contribute to gametophytic cell fate differentiation.

Based on an experiment by Saze *et al.* 2003[10] we exclude the non-random segregation of whole chromatids in mitosis 1-3 during *Arabidopsis* female gametophyte development. Mutations in *Arabidopsis thaliana* that cause a loss of function in MET1, a protein responsible for the semiconservative replication of CpG DNA methylation marks, are recessive. Since the development of the *Arabidopsis thaliana* female gametophyte includes 3 postmeiotic haploid cell divisions, in *met1* mutant lines only 25% of the resulting 8 nuclei should contain hemimethylated DNA whereas 75% should include fully demethylated DNA if chromatid segregation is random (Figure 8). Saze *et al.* 2003[10] confirmed the random distribution by measuring the expression of an epigenetically silent and hypermethylated locus consisting of several copies of the β-glucuroindase reporter gene (*GUS*). They did not correlate these results with the segregation of chromatids meaning this information is new. Although these results exclude the non-random segregation of the chromatid region bearing the studied locus during *Arabidopsis thaliana* gametophyte development, it does not entirely reject the hypothesis of non-random sister chromatid segregation. Homologous sister chromatid

exchanges (SCE) during cell division could re-randomize non-randomly distributed chromatids. The silent sister hypothesis (SSH)[11] equals the SSIS hypothesis, but assumes not the non-random segregation of whole chromatids but rather only some specific chromatid regions, mainly chromatid centromeres.

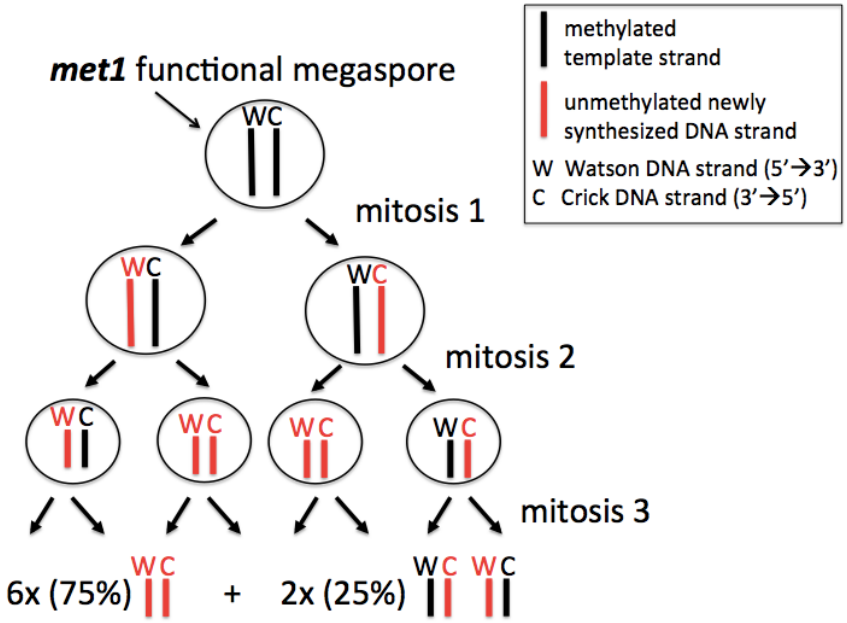

Figure 8 Random segregation of sister chromatids in the *Arabidopsis thaliana* female gametophyte. *MET1* is responsible for the semiconservative replication of CpG methylation marks during cell division. In a line heterozygous for the *met1* mutation DNA methylation is intact in diploid cells and hence the diploid megaspore mother cell contains two regularly methylated chromatids. However, after meiosis, the chance is 50% that the functional megaspore is homozygous for *met1*. In this case, semiconservative replication of CpG methylation does not occur anymore. As a consequence, all newly synthesized DNA strands during mitosis 1-3 bear no CpG methylation marks and hence the template strand is specifically marked with CpG methylation marks. The results published by Saze *et al.* 2003[10] point to a random segregation of the non-methylated template strands.

Sister chromatid exchanges: Non-random segregation of the chromatid centromeres

SCEs often occur after replication blockage at sites of DNA damage. Many environmental factors like UV light, toxins as well as endogenous factors like reactive oxygen species generated by normal cellular metabolism can cause DNA damage and block DNA replication. DNA replication blockage leads to stalled replication forks, which are inherently unstable posing a serious threat to genomic integrity[12]. Depending on the kind of DNA damage, different repair mechanisms are involved in restarting stalled replication forks. Some of those mechanisms include homologous sister chromatid exchange. Sister chromatid exchanges during replication lead to a fusion of the damaged template strand with the newly synthesized strand based on the other template strand of the same chromatid (Figure 9). Mixing template strands with newly synthesized strands re-randomizes non-randomly segregated chromatids.

If SCEs are assumed to occur randomly with the same frequency along the whole length of a chromatid, the chance of two loci to be separated is higher the more distant they are. Kinetochores consist of a protein complex and connect microtubules with the centromeres. Since microtubules attach to the centromeres via the kinetochores to separate and distribute the sister chromatids, chances are higher to lose more distant parts towards the telomeres. We assume that at centromeres, chromatids are rarely mixed by SCEs and segregate non-randomly during non-random sister chromatid segregation. Non-random segregation of only the centromere would imply that the chromatid specific epigenetic markers locate in the centromere region, which is following the SSH model.

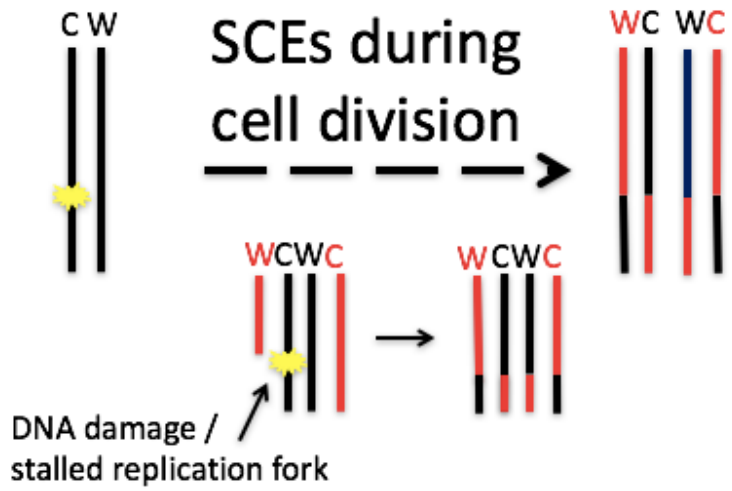

Figure 9 SCEs re-randomize non-random segregating chromatids. DNA replication stops at regions of DNA damage (yellow star). A DNA damage located on the template (black) Crick strand leads to a stalled replication fork and stops the synthesis of the newly synthesized (red) Watson strand. One mechanism to repair the causing DNA damage and to restart the replication fork is homologous sister chromatid recombination. The DNA damage located either on the Watson or the Crick strand of one chromatid is repaired by reading the intact strand from the other sister chromatid. Homologous sister chromatid recombination, therefore, requires the fusion of the damaged template (black) Crick strand with the undamaged newly synthesized (red) Crick strand from the other chromatid.

WYR: A candidate gene for the regulation of sister chromatid segregation

Non-random DNA segregation must involve asymmetric proteins, which confer the non-random segregation.

In budding yeast both the kinetochore protein Ndc10[13] and Sli15 (Chapter 2), which is required for Ndc10 recruitment, segregate asymmetrically during mitosis. Furthermore, the microtubule organizing centers; human centrosomes[14], *Drosophila* centrosomes[15] and budding yeast spindle pole bodies[16] segregate non-randomly. One cell type always receives the older centrosome whereas the other cell type receives the younger centrosome.

Kinetochore proteins and centrosomes are essential for the correct segregation of chromatids to both daughter cells. We propose that their asymmetric distribution might cause an asymmetric and non-random segregation of sister chromatids. *WYR*, the plant

ortholog of yeast *SLI15*, plays a role in the *Arabidopsis thaliana* female gametophytic cell specification and hence might link non-random sister chromatid segregation with cell specification[17].

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Chapter 2 – Method Development

Method development for strand-specific single cell sequencing

A method for strand-specific single cell sequencing has been published by Falconer et al. 2012[1]. This method has been developed and optimized for flow sorted single mammalian embryonic stem cells from cell cultures. The incorporation of the nucleotide analog 5-Bromo-2'-deoxyuridine (BrdU) to label newly synthesized DNA strands during one mitosis was controlled by growing cell cultures for a determined period in a nucleotide analog containing medium. The exact incubation time required to label synchronized cells during one mitosis was determined by immunodetection of BrdU in a test culture.

This procedure is not ideal to study sister chromatid segregation in multicellular tissues like the male gametophyte of *Arabidopsis thaliana*. Cells can't be synchronized, nucleotide analog uptake through the vascular tissue might differ from inflorescence to inflorescence and the nucleotide analog can't be removed immediately by washing cells and replacing the medium. Accordingly, I had to adapt the method.

5-Ethinyl-2'-deoxyuridine (EdU): An alternative to 5-Bromo-2'-deoxyuridine (BrdU)

To determine for each analyzed cell individually how many mitoses the nucleotide analog has been incorporated, I was looking for an alternative nucleotide analog, which is detectable without the need of destroying the sample, as necessary for immunodetection.

5-Ethinyl-2'-deoxyuridine (EdU) is a nucleotide analog detectable by click chemistry and does not require the sample to be destroyed. However, EdU is not sensitive to UV-light

irradiation and strands containing EdU can't be removed by the same procedure as applied for BrdU. Therefore, I replaced the UV-light based introduction of abasic sites with an enzymatic reaction. Mismatch-specific DNA glycosylase (MUG) has been described to recognize different nucleotide analogs like BrdU and to produce abasic sites[2]. I could show that MUG recognizes and excises EdU to create an abasic site. As for BrdU, Exonuclease III recognizes these abasic sites and digests all DNA strands, which contained EdU.

Isolation of single pollen nuclei from one pollen grain

To analyze sister chromatid segregation during single pollen development, I tried to isolate single pollen nuclei by either micromanipulation or laser capture microdissection. For both methods, I had to lyse pollen to release all 3 nuclei. Laser capture microdissection of intact pollen was not successful because the laser burned the pollen wall and destroyed the sample. Also, the nuclei inside intact pollen are too close to be separated by laser capture microdissection. I tried to lyse pollen with 3 methods: enzymatic pollen wall digestion, pollen squeezing, and mechanical glass bead disruption. Enzymatic pollen wall digestion and pollen squeezing failed mainly when I washed pollen with deionized water. This step, which often leads to osmotic cell lysis, is necessary because salts and sugar on the dry microdissection slides disturb the laser during laser capture microdissection.

Finally, I could successfully lyse pollen by mechanical glass bead disruption modified according to Borges *et al.* 2012[3], who lysed pollen to isolate single nuclei by fluorescence activated cell sorting (FACS). However, their method is not able to assign the isolated nuclei to the pollen of origin and therefore it is unknown which vegetative and generative nuclei stem from the same pollen. As I found out, the size of the glass beads

determines whether the 3 nuclei are separated or not (Figure 1). Small glass beads (0.5mm) used by Borges *et al.* 2012[3] physically disrupt the cytoplasmic connection[4] between the three pollen nuclei, whereas the bigger glass beads (5-7mm) don't.

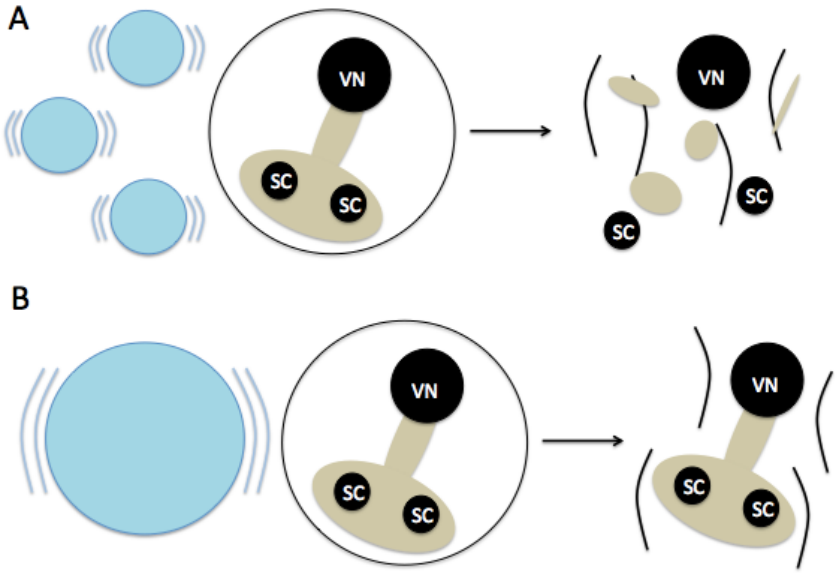

Figure 1 Mechanical pollen lysis by glass beads. **A)** Smaller glass beads (0.5mm) destroy the cytoplasmic connection between all 3 nuclei (VN = vegetative nucleus, SC = sperm cell). **B)** Bigger glass beads (5-7mm) don't separate the three nuclei.

I lysed pollen in sperm extraction buffer[3], washed them in water, pipetted them on to a microdissection slide, analyzed them for the position of intact male germ units with an epifluorescence microscope, air dried them, fixed them with hair spray and cut them by laser capture microdissection. Finally, I controlled the presence of the nucleus with an epifluorescence microscope (Figure 2).

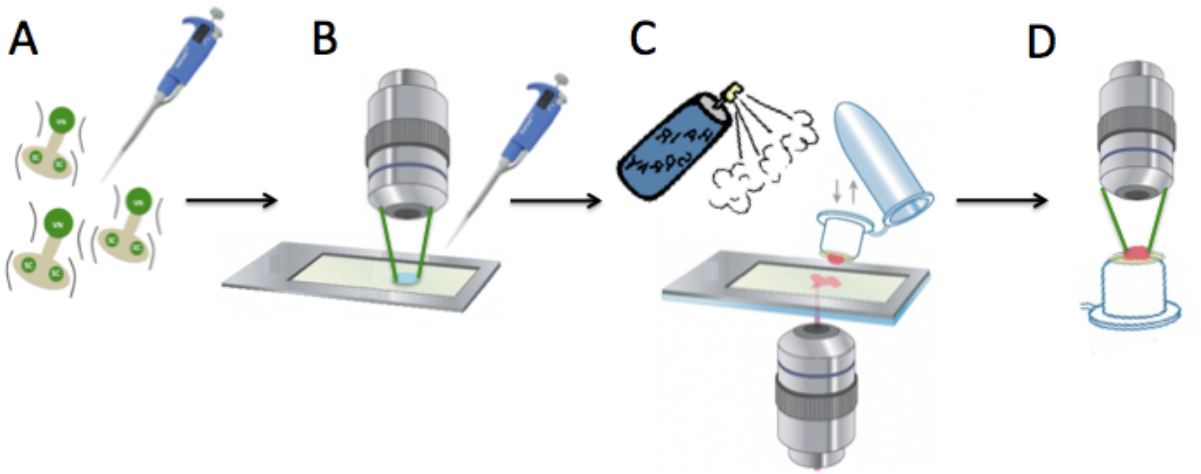

Figure 2 Laser capture microdissection of single pollen nuclei. **A)** Lysed pollen washed with deionized water to remove salts and sugars. **B)** Lysed pollen pipetted in small droplets (ca. 1uL) onto a laser capture microdissection slide and analyzed for EdU-6-Carboxyfluorescein- or DAPI fluorescence under the epifluorescence microscope. **C)** Air-dried slides are fixed with hair spray and single nuclei cut with a laser capture microdissection microscope. The cut region sticks to the lid of a laser capture microdissection isolation tube. **D)** Isolated samples are stored at -80°C. After removing the samples from the freezer, the condensation water allows checking the presence of the fluorescence from isolated nuclei with an epifluorescence microscope.

Pilot experiment 1: Evaluation of feasibility

In a pilot experiment I tried to follow the procedure described by Falconer *et al.* 2012[1]; enzymatic fragmentation of single cell genomic DNA, direct purification of fragmented single cell DNA by ethanol precipitation followed by strand-digestion, DNA purification and library preparation (Figure 3A). I prepared 3 barcoded libraries; 2 libraries each from a single pollen sperm nucleus and one barcoded water control library. In one sample I digested all EdU containing DNA strands with both enzymes MUG and Exonuclease III. The other sample served as a negative control, and I did not digest EdU containing DNA strands. As suggested by the Functional Genomics Center Zürich, where I prepared the libraries, I used the Nugen Ovation Ultralow kit to make the libraries. The libraries were run on one Illumina Hi-Seq 2500 lane together with other barcoded samples. For the sample treated with MUG and ExoIII, I could map 1914 reads to 12 regions of the *Arabidopsis thaliana* genome (Figure 4A). For the sample not treated with

MUG and ExoIII I could map 2918 reads to 40 regions of the *Arabidopsis thaliana* genome (Figure 4B). For the water control I could map 126 reads to 4 regions of the *Arabidopsis thaliana* genome (Figure 4C). Altogether, the results indicated that the protocol works; highest number of reads in the sample where I did not digest EdU-constituted strands, and almost no reads in the water control. However, I had not enough reads to track sister chromatid segregation. I could also not increase the number of reads by deeper sequencing (loading more sample).

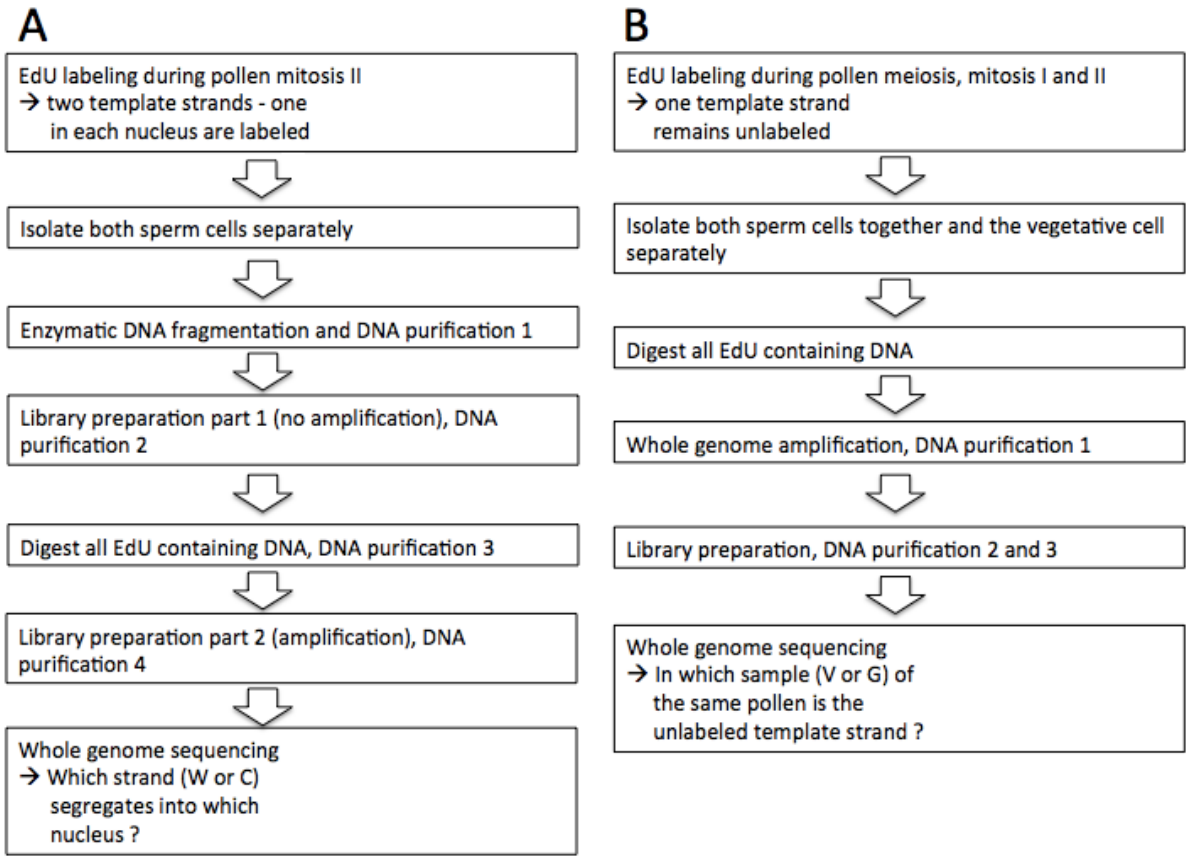

Figure 3 Flowchart of procedures to track sister chromatid segregation during pollen development. **A)** Procedure to track sister chromatid segregation similar to the procedure published by Falconer *et al.* 2012[1], except that I used EdU instead of BrdU nucleotide analog and an enzymatic reaction instead of UV-light to produce abasic sites at positions of EdU substitution. Genome coverage was always low and contamination relatively high. **B)** Second and favored method to track sister chromatid segregation. All steps after laser capture microdissection until and inclusive whole genome amplification are performed in the same tube without any DNA purification step. This allows reducing both, the loss of single cell DNA and the introduction of contamination. DNA fragmentation is not necessary due to whole genome amplification with an average amplicon size of 500 base pairs.

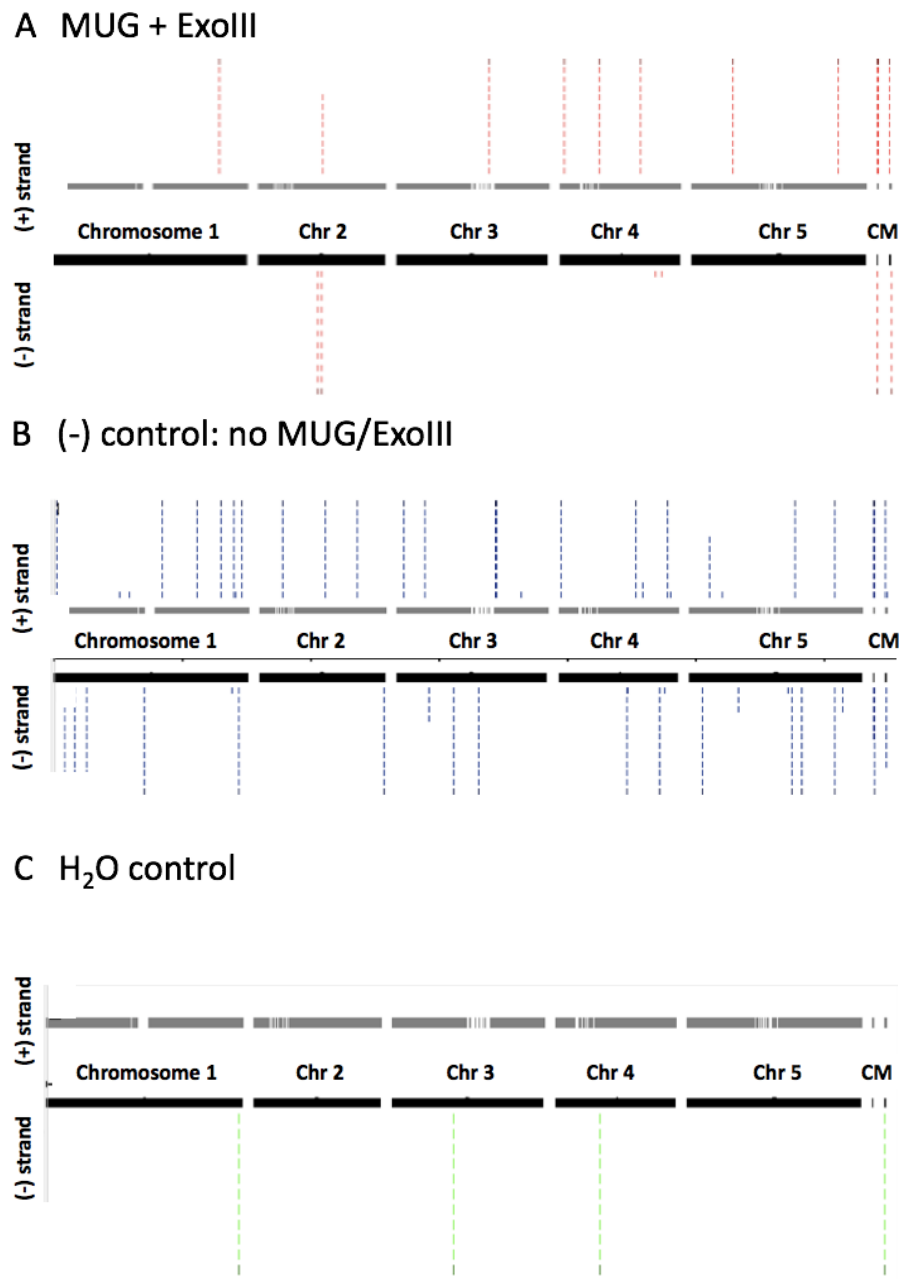

Figure 4 Pilot Experiment 1: Sequencing results. Procedure: See Figure 3A. Black bars: chromosomes, gray bars: gene expression. C: chloroplast DNA. M: mitochondrial DNA. **A)** Single sperm cell treated with MUG and Exonuclease III to digest EdU-substituted DNA strands. **B)** Negative control: No MUG nor Exonuclease III added. **C)** H_2O control (no input DNA)

Pilot experiment 2: Improvement of genome coverage

To improve the genome coverage and to reduce the loss of genomic DNA I started a test series (Table 1) with the protocol from pilot experiment 1. I tried different incubation times for the fragmentation of the single cell genomic DNA to prevent the production of

too short or too long fragments and I also replaced the first DNA purification step by ethanol precipitation with DNA purification with magnetic beads. Different to the first pilot experiment, I prepared the libraries on my bench and not at the Functional Genomics Center. Accordingly, I used reagents and consumables (library preparation kit, magnetic beads, pipette tips, tubes, …) not from the Functional Genomics Center. The goal of pilot experiment 2 was to increase the number of reads and the genome coverage. I collected random single sperm nuclei from different pollen.

Magnetic beads	EtOH precipitation
20' fragmentase	20' fragmentase
40' fragmentase	40' fragmentase
80' fragmentase	80' fragmentase
3h fragmentase	3h fragmentase
o/n fragmentase	o/n fragmentase

Table 1 Libraries prepared to increase genome coverage/number of reads. For this run, I prepared 5 libraries where I purified fragmented single cell gDNA with magnetic beads and 5 libraries where I purified fragmented single cell gDNA by ethanol precipitation. For both purification methods, I produced libraries where I fragmented single cell gDNA during 20min, 40min, 80min, 3 hours or overnight.

I could successfully increase the genome coverage for all samples purified with magnetic beads and only for some samples purified by ethanol precipitation (Figure 5). From these results, I interpreted that magnetic beads are more efficient for the purification of fragmented single nucleus genomic DNA. However, the reads mapped equally to both strands, the (+) and (-) strands. When I mapped the number of reads mapped to the (+) and (-) strand separately for each read depth (here number of PCR replicates) I observed an asymmetric distribution of the reads to the (+) and (-) strand for reads with read depth 1 (Figure 6). I interpreted that these are the sequences containing EdU-6-Carboxyfluorescein (EdU-FAM), which were not digested but also not amplified because 6-Carboxyfluorescein (FAM) is a DNA-polymerase inhibitor. To filter asymmetric reads, I wrote an AWK script, which filters for asymmetric reads. The script builds on the fact

that symmetric reads coming from an intact dsDNA fragment result in two reads mapped nearby on opposite DNA-strands whereas asymmetric reads from a ssDNA fragment where one strand has been digested lead to only one read mapped either to the (+) or (-) strand (Figure 7). This AWK script isolated reads in which proximity no other reads mapped on the opposite strand. I tested my script with publicly available raw data from Falconer *et al.* 2012[1] (Figure 8).

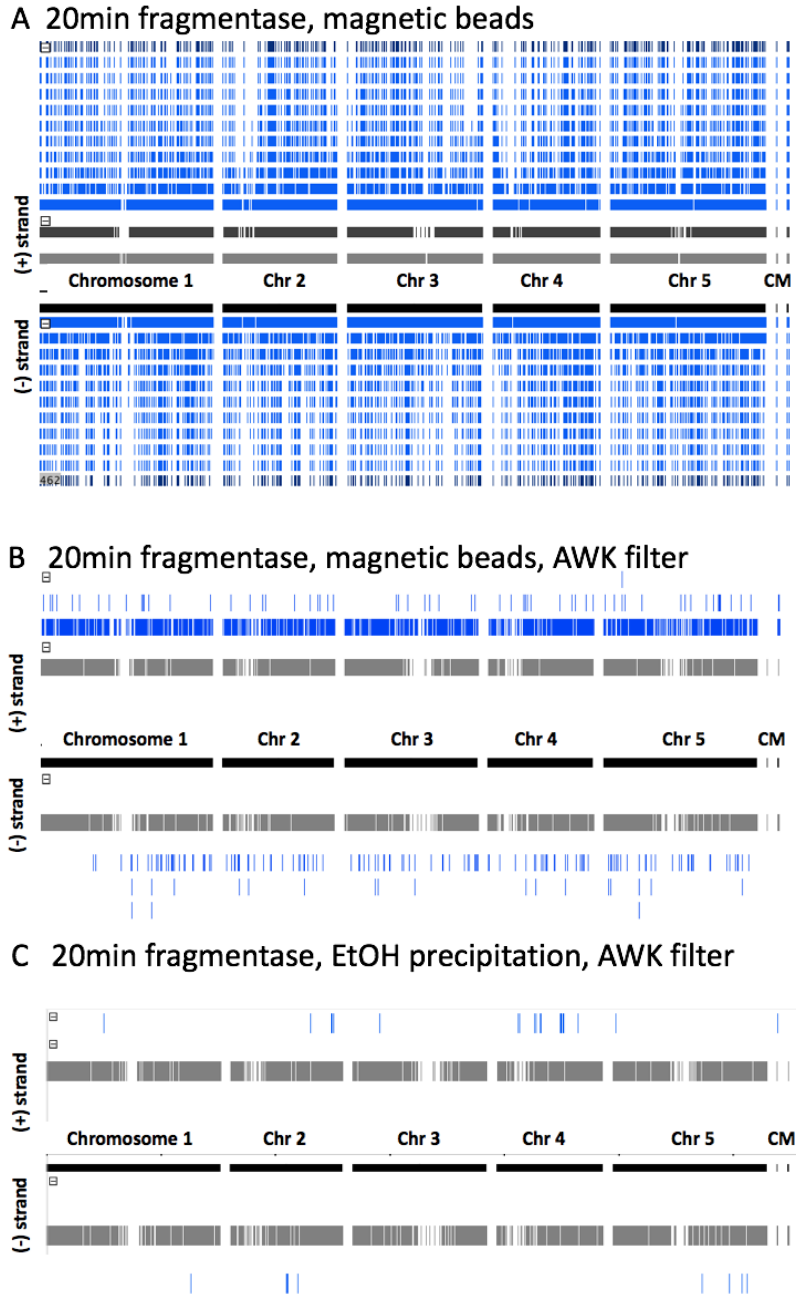

Figure 5 Pilot Experiment 2: Sequencing results. Procedure: See Figure 3A and Table 1. **A)** Reads mapped to genome of *Arabidopsis thaliana* from single sperm genomic DNA fragmented for 20min with fragmentase and purified with magnetic beads. **B)**

Same sample as in A) but filtered for asymmetric reads (Figure 7). **C)** Reads mapped to the *Arabidopsis thaliana* genome from single sperm genomic DNA fragmented for 20min with fragmentase and purified by ethanol precipitation.

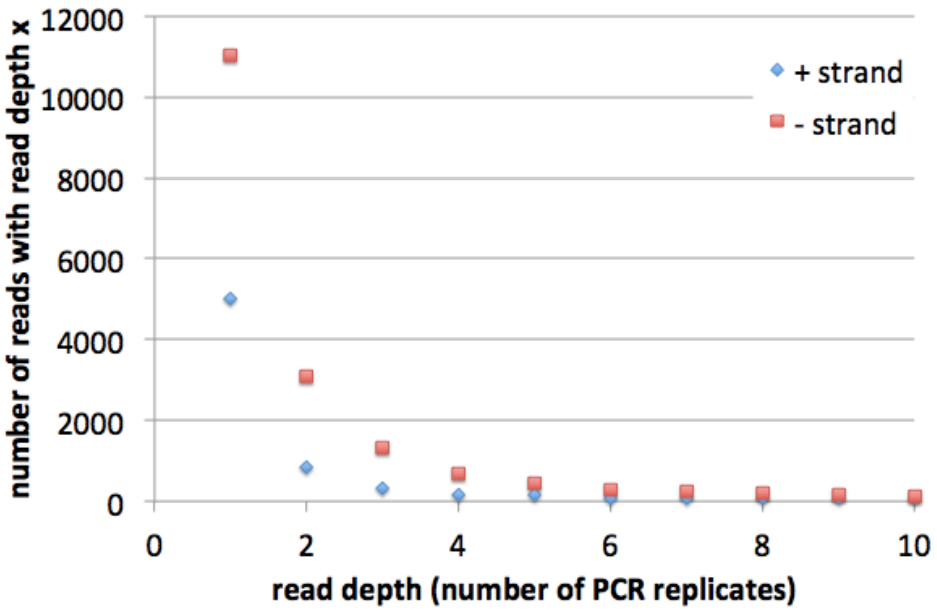

Figure 6 Read depth frequency for reads mapped to the (+) and (-) DNA strands. For reads with read depth 1, there is an accumulation of reads mapped to the (-) strand compared to reads mapped to the (+) strand.

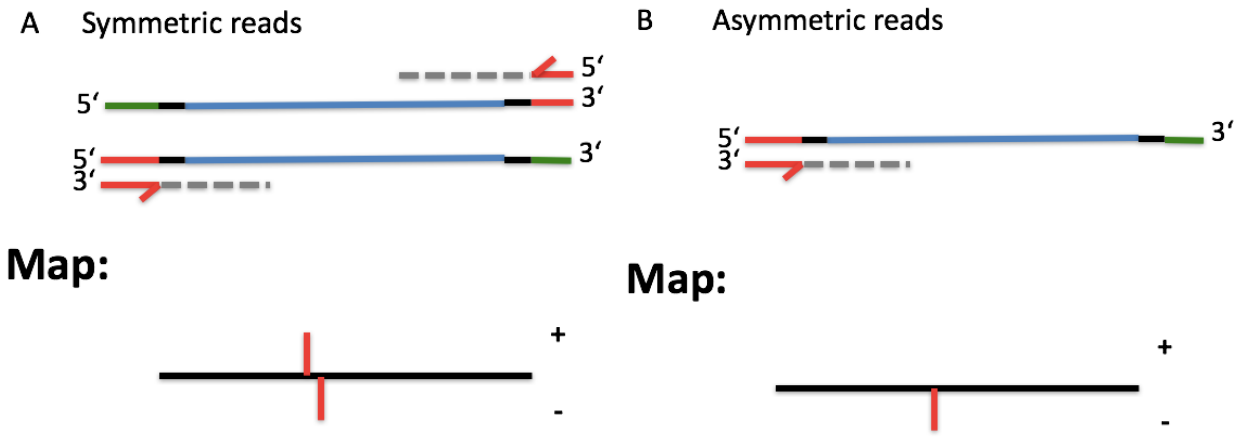

Figure 7 Expected mapping results for symmetric and asymmetric reads. **A)** No EdU containing undigested DNA fragments result in two reads nearby on opposite DNA-strands. **B)** EdU containing digested DNA fragments lead to only one read on one DNA strand.

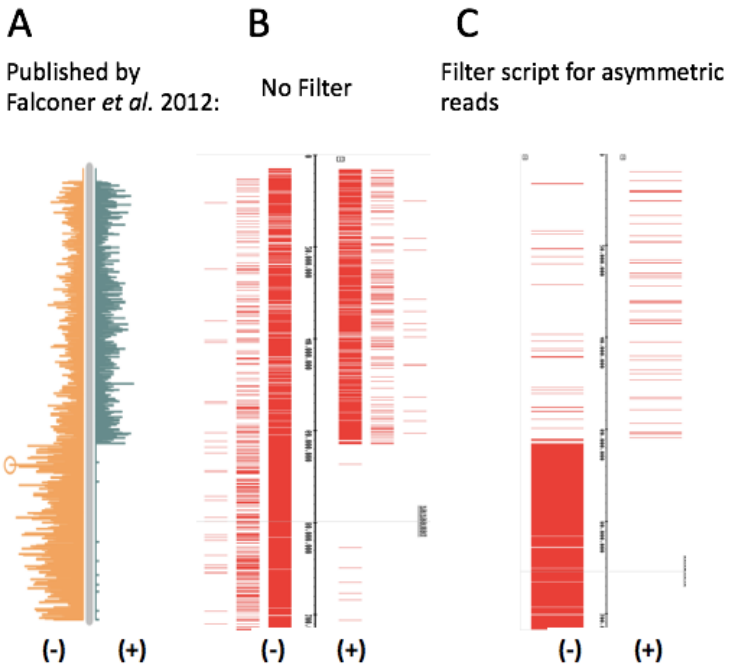

Figure 8 Test of the AWK script to isolate asymmetric reads. **A)** Sequencing data mapped to both (-) and (+) strand by Falconer *et al.* 2012[1]. The upper part contains symmetric reads (reads map to both strands) whereas the lower part contains asymmetric reads (reads map only to the (-) strand). **B)** Mapping results for downloaded publicly available raw data from Falconer *et al.* 2012[1] mapped with Bowtie 2.0. Results are similar to A). **C)** Mapping results for asymmetric reads isolated with my AWK script. My script discarded most of the symmetric reads in the upper part but kept the asymmetric reads in the lower part.

Altogether, the results pointed to an efficient purification of fragmented single nucleus DNA with magnetic beads. The digestion of EdU-containing strands seemed not to be complete, but I could separate asymmetric reads from symmetric reads with an AWK script.

Experiment 1: Procedure A

Due to the results of pilot experiment 2, I decided to start a first experiment to track sister chromatid segregation during pollen mitosis 2. I isolated both sperm nuclei from single pollen separately. By incorporating EdU during pollen mitosis 2 only (Figure 9) I expected to sequence the opposite strands in both sperms origination from the same pollen (Figure 9 and Figure 10A). I prepared one barcoded library for each sperm

nucleus from 4 pollen (= 8 libraries), 6 libraries from sperm nuclei, which were not labeled with EdU and one water control library.

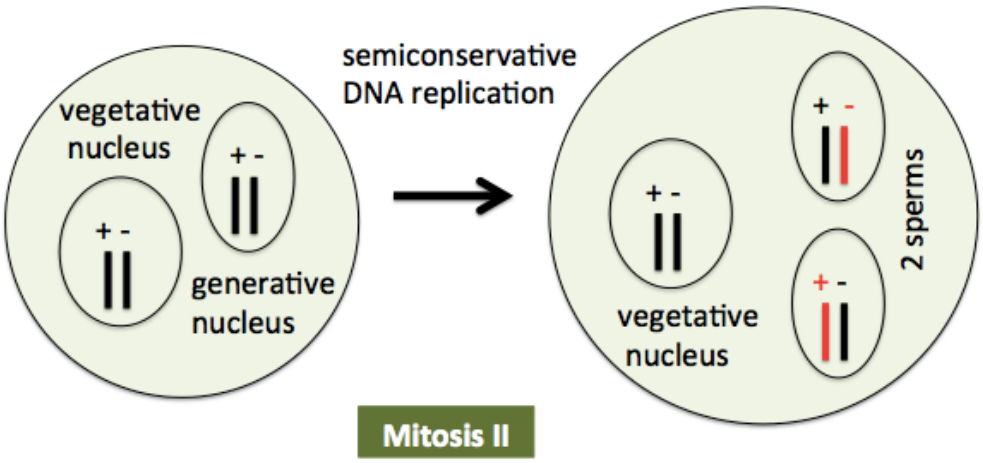

Figure 9 EdU incorporation during pollen mitosis 2. Incorporation of EdU during pollen mitosis 2 leads to two sperm nuclei containing chromosomes with EdU-substitution either in the (+) or the (-) DNA strand but always in the opposite strand for both nuclei.

Unfortunately, the genome coverage was much lower than in pilot experiment 2, and I did not get the expected result of reads mapping to opposite strands for both sperm nuclei originating from the same pollen (Figure 10B). In addition, most reads seemed not to be asymmetric. In pilot experiment 2 I used an open batch of magnetic beads. In this experiment, I used a new batch of magnetic beads but prepared a water control for both the old batch of magnetic beads used by other lab members and the new batch of magnetic beads. The old batch of magnetic beads contained a lot of *Arabidopsis thaliana* gDNA contamination (Figure 11).

With these results, it became clear that I could not significantly increase the genome coverage and that most of the reads are contamination.

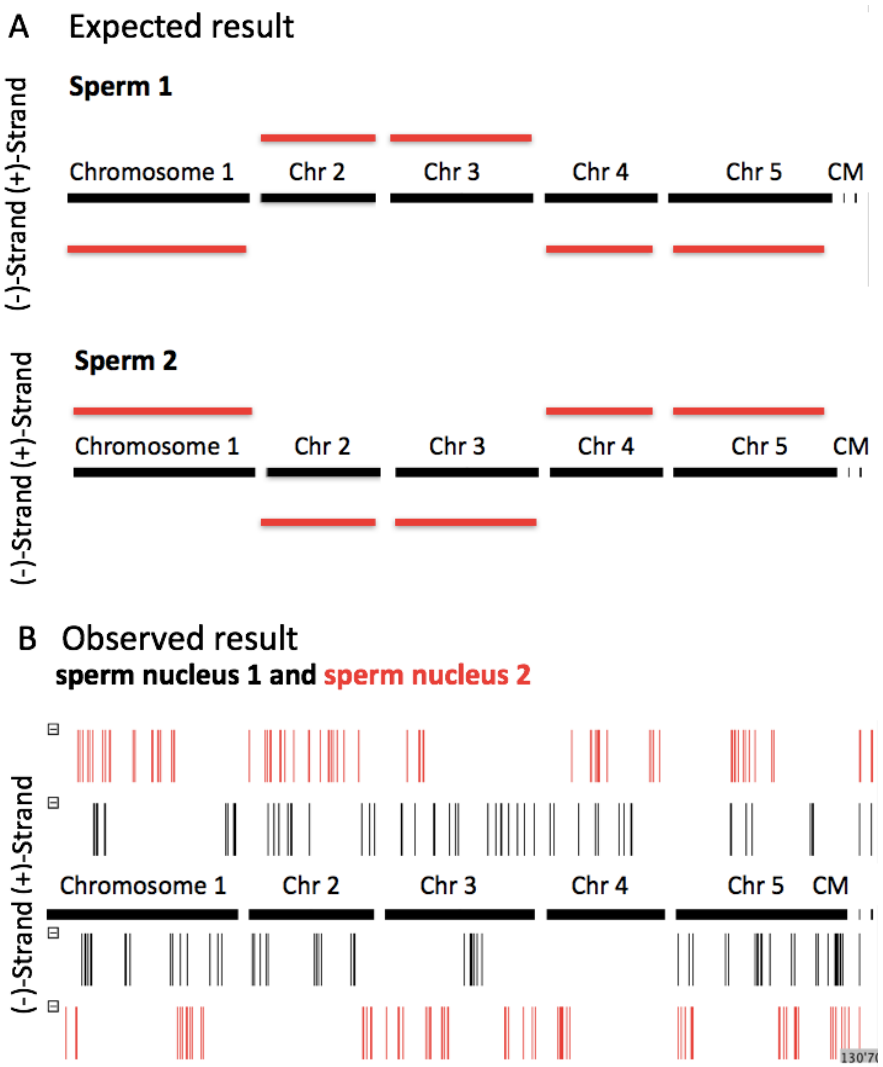

Figure 10 Experiment 1: Sequencing results. **A)** Expected results: If EdU was incorporated during pollen mitosis 2 only I would expect to observe reads mapping to one strand from sperm one (in this example to the (-) strand for chromosome 1) and reads mapping to the opposite strand from sperm 2 (in this example to the (+) strand for chromosome 1). **B)** Observed results: reads from sperm 1 (black) often map to the same strand as reads from sperm 2 (red). Compared to pilot experiment 2 (Figure 5) most reads are not asymmetric anymore, and the genome coverage is much lower.

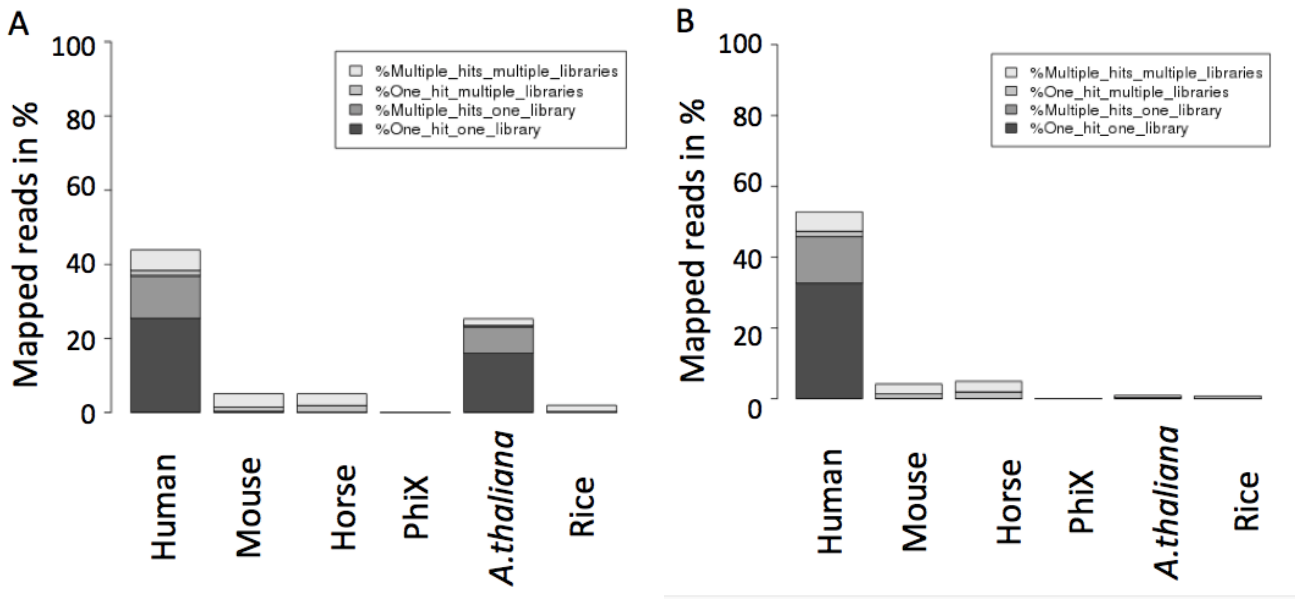

Figure 11 Water control: Pilot Experiment 2 and Experiment 1: FastqScreen. **A)** The old open batch of magnetic beads used for pilot experiment 2 contains a lot of *A. thaliana* contamination. **B)** The new batch of magnetic beads contains much less *A. thaliana* contamination.

Experiment 2: Procedure B

Pilot experiments 1 and 2 and experiment 1 according to procedure A (Figure 3A) failed either due to a low genome coverage or DNA contamination. At this point, I decided to modify the protocol according to procedure B (Figure 3B). Procedure A requires 3 steps of single cell genomic DNA purification before library amplification. The purification of as little DNA as from a single haploid cell poses high risks of losing DNA and introducing contamination. Also, most low DNA input library kits are designed for DNA amounts of more than 1ng. One haploid nucleus of *Arabidopsis thaliana* contains about 0.4pg of DNA – 2500x less DNA than required. The excess of adaptor sequences in the ligation step during library preparation tends to produce contamination and adapter dimers. The dilution of adaptor sequences to such tiny amounts is risky as well. In literature (reviewed in Gawad *et al.* 2016[5]) most single cell DNA sequencing methods include a step of whole genome amplification after single cell isolation and before DNA purification and library preparation. Some whole genome amplification kits are

designed to amplify DNA from single cells. DNA from a single cell is amplified directly after cell lysis without prior DNA purification and without changing the tube. After whole genome amplification, the amplified DNA from a single cell can be purified without the risk of losing significant parts of total DNA and without the risk of introducing heavy contamination. After whole genome amplification, the amount of DNA meets the requirements of low input library preparation kits. The drawback of whole genome amplification for strand-specific DNA sequencing is the loss of information about strandnes. A dsDNA fragment containing one EdU-unlabeled and one EdU-labeled strand (Figure 9) incubated with MUG and Exonuclease III to digest the EdU-labeled strand and to produce a ssDNA fragment can't be distinguished from a dsDNA fragment with two EdU-unlabeled strands. The information about which DNA-strand(s) served as template strand is lost.

However, it's possible to distinguish whether both (2) or fewer template strands of a dsDNA fragment were EdU-labeled. If both strands were labeled, both would be digested and nothing would be amplified and sequenced. If one or both stands were EdU-unlabeled, the fragment would be amplified and sequenced. Accordingly, I incorporated EdU during pollen meiosis, pollen mitosis 1 and pollen mitosis 2 (Figure 12). For a particular fragment, this produces 1 nucleus with 1 EdU-unlabeled DNA strand, which is amplified and sequenced and 2 nuclei with 2 EdU-labeled DNA strands, which are digested and can't be sequenced. Therefore, only 1 nucleus contains an EdU-unlabeled DNA strand, which is sequenced. With that experiment design, I wanted to study the segregation of this only EdU-unlabeled DNA strand. Does it randomly segregate to the vegetative nucleus and the generative nucleus during pollen mitosis 1 or is there a bias for one of both cell types?

Procedure A can distinguish between the random/non-random segregation of the (-) or (+) strand during pollen mitosis 2. Procedure B can make a distinction between the random/non-random segregation of the oldest DNA strand during pollen mitosis 1. Experiment 2 was successful, and I could track the segregation of the oldest DNA template strands. Results are described in Chapter 3.

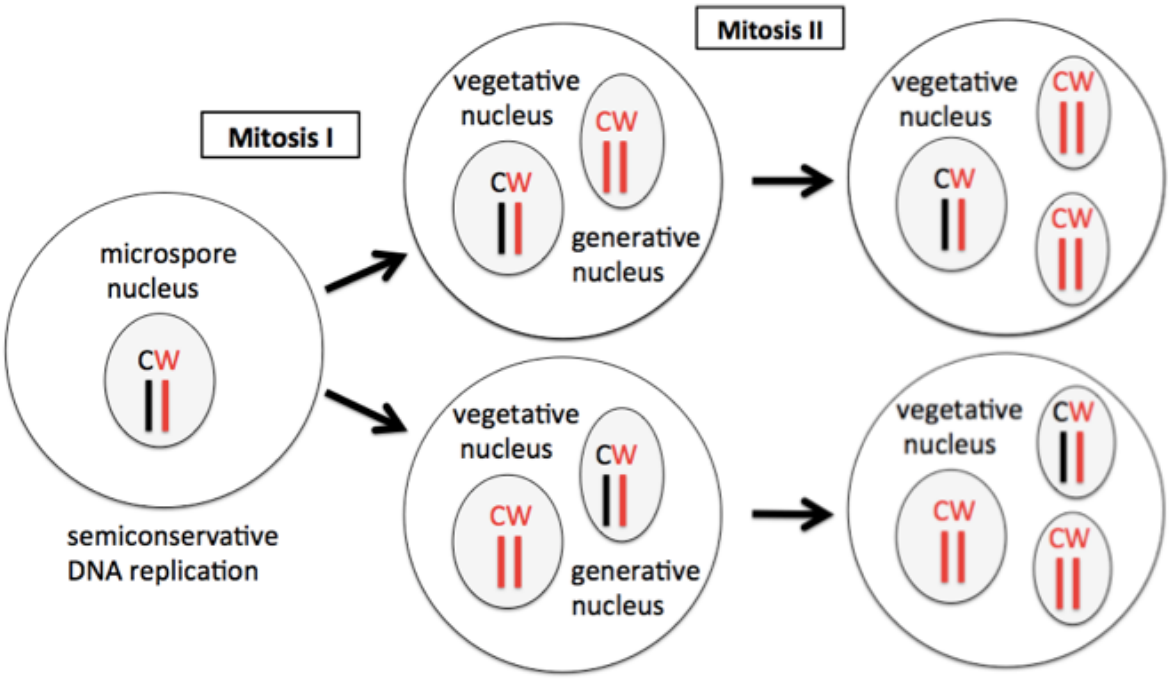

Figure 12 Experiment design for procedure B. To follow procedure B (Figure 3), which can distinguish between dsDNA fragments with both DNA strands substituted with EdU (red) and dsDNA fragments with either 1 or both DNA strand not substituted with EdU (black), I incorporated EdU during pollen meiosis, mitosis 1 and mitosis 2. Looking at one specific dsDNA fragment, pollen mitosis 1 produces two nuclei of which one nucleus contains only EdU-labeled DNA strands and one nucleus, which contains the oldest EdU-unlabeled DNA strand. In experiment 2 I tracked the segregation of the only EdU-unlabeled DNA strand to either the vegetative or generative nucleus.

Experiment 3: Evaluation of reproducibility of experiment 2

I repeated experiment 2 with fresh plant material and could successfully reproduce the results of experiment 2. The results are described in Chapter 3.

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Chapter 3 – Manuscript 1

Introduction

Cell division, the fundamental process of any biological reproduction, ensures genome integrity by semiconservative DNA replication and equal chromosome segregation. However, the inherent characteristic of replication to produce replication mistakes leads to an accumulation of replication errors during consecutive cell divisions.

First, in bacteria[1] and later in mammalian[2] and plant[3] cells Lark observed the coordinated segregation of sister chromatids: One cell inherits all the template strand containing chromatids, whereas the other cell inherits all the newer strand containing chromatids (Figure 1A). Based on that observations Cairns 1975[4] came up with the "immortal strand" hypothesis: Stem cells protect themselves from replication errors by the coordinated and directed segregation of sister chromatids. Among all chromosomes the oldest DNA strand containing chromatids with the least replication errors segregate to the stem cell fate retaining daughter cell whereas chromatids containing the newer DNA strands segregate to the differentiated daughter cell.

With a new method (Figure 1B), DNA strand age-specific single cell sequencing, we could confirm the existence of significant coordinated sister chromatid segregation during *Arabidopsis thaliana* pollen meiosis and mitosis 1 (Figure 1A). Coordinated sister chromatid segregation is one of two essential conditions to accept the immortal strand hypothesis and has to our knowledge never been tested at a genome-wide level in any metazoan.

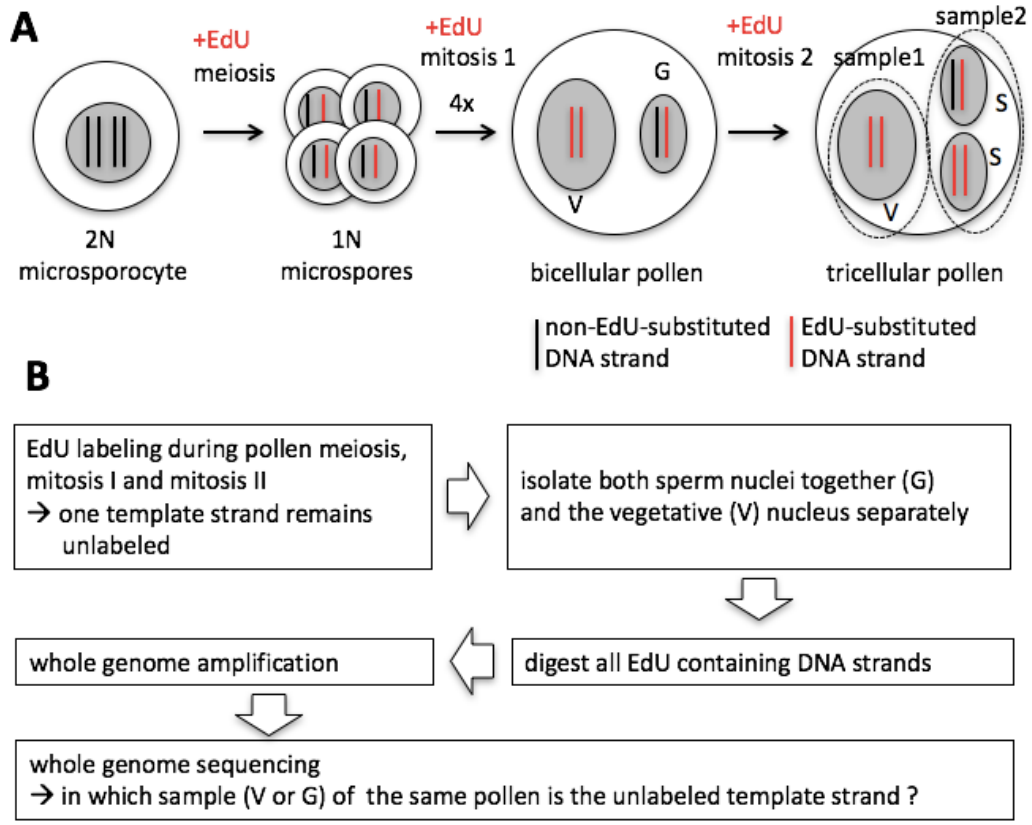

Figure 1 5-Ethynyl-2'-deoxyuridine (EdU) incorporation during pollen development and strand-age specific sequencing. **A)** *Arabidopsis thaliana* pollen development. A diploid microsporocyte enters meiosis to form 4 haploid microspores. EdU incorporation during premeiotic S-phase results in chromatids consisting of one EdU-substituted DNA strand (red) and one non-EdU-substituted (black) template strand. During asymmetric pollen mitosis 1, microspores divide to form bicellular pollen composed of a vegetative and a generative cell. EdU substitution during both S-phases before meiosis and mitosis 1 leads to 5 chromosomes each consisting of one chromatid with 2 EdU-substituted DNA strands and one chromatid with one substituted and one non-substituted strand. According to the immortal DNA hypothesis, the segregation of chromatids containing the non-EdU-substituted DNA strand should be coordinated and directed. To track the segregation of the non-EdU-substituted strands into either the vegetative or generative nucleus, we collected the vegetative nucleus (sample 1) and the two sperm nuclei (sample 2) separately. **B)** Strand-age specific sequencing method (Figures S1-S5). To label newly synthesized strands according to figure 1A, we grew cut inflorescences for 8 days in EdU containing growth medium, fluorescently labeled EdU with 6-Carboxyfluorescein by click-chemistry and isolated samples 1 and 2 from single pollen by laser-capture microdissection. Isolated nuclei were lysed and treated with the enzymes T/U mismatch-specific DNA glycosylase (MUG) and Exonuclease III to remove all EdU-substituted DNA strands. Non-substituted DNA was amplified by whole genome amplification and sequenced by Illumina HiSeq 2500.

Results

Two essential conditions must be fulfilled to accept the immortal strand hypothesis: The existence of coordinated and directed sister chromatid segregation. Mainly the lack of methods to track sister chromatid segregation due to technical difficulties has lead to conflictive results[5]. Here, with a new method (Figure 1B), we tested whether both conditions of coordinated and directed sister chromatid segregation are fulfilled during *Arabidopsis thaliana* pollen development (Figure 1A). During microsporogenesis, the first step of pollen development, a diploid microsporocyte enters meiosis to form 4 haploid microspores. Each of these microspores undergoes asymmetric pollen mitosis 1 to form a vegetative and a generative cell and finally the generative cell undergoes pollen mitosis 2 to form 2 sperm cells. We analyzed the segregation of the oldest DNA strand of all 5 chromosomes by labeling all newly synthesized DNA strands during pollen meiosis, mitosis 1 and mitosis 2 with 5-Ethynyl-2'-deoxyuridine (EdU), a nucleotide analog (Figure 1A and S2A-B). By laser capture microdissection (Figure 1A and S2C) we isolated two samples: One sample containing the vegetative nucleus and the other sample containing both sperm nuclei of single mature pollen (Figure 1A and S2C). To assign the segregation of all 5 non-EdU-substituted strands, which served as the template strands during pollen meiosis, to either the vegetative nucleus or one of both sperm nuclei, we digested newly synthesized EdU-substituted DNA with *E. coli* T/U mismatch-specific DNA glycosylase (MUG) and Exonuclease III (Figure S1 and S4). Upon whole genome amplification, the presence of non-digested template strands in both samples was analyzed by Illumina HiSeq 2500 whole genome sequencing. To exclude randomization of the results by meiotic recombination (Figure 4) we considered only reads mapped to the centromeric regions. In two independent experiments with a total of 17 pollen and 34 samples we found evidence for the coordinated segregation of

all 5 premeiotic template centromeres in about 50% (10/16) of all analyzed pollen (Figure 2 and S6): Among all chromosomes the premeiotic DNA template strand containing chromatids segregate to the same daughter cell, either the vegetative or generative nucleus of pollen. However, the direction of segregation is random (Figure 2 and S6): In that 50% of all pollen with coordinated sister chromatid segregation the premeiotic DNA template strands segregate together randomly either into the vegetative or generative nucleus. 9 pollen were significant for the cosegregation of centromeres (Figure S9B), exact binomial test for goodness-of-fit, $P<0.05$.

	A1	A2	A3	A4	A5	A6	A7	A8	A9	B4	B5	B6	B7	B8	B9	B10	B11	G:V
Chr1	$M^{2:1}$	$M^{2:1}$	$G^{5:0}$	$G^{3:1}$	$G^{1:0}$	$G^{3:1}$	$V^{0:1}$	$V^{0:11}$	0	?	$V^{0:2}$	0	$V^{0:2}$	0	-	$V^{1:164}$	$V^{0:4}$	4:6
Chr2	$G^{2:0}$	0	$G^{6:0}$	$G^{5:0}$	$M^{2:1}$	$M^{2:1}$	$G^{1:0}$	$V^{1:4}$	0	?	$V^{1:5}$	$V^{0:1}$	$V^{0:2}$	$G^{1:0}$	-	$V^{0:53}$	$V^{0:2}$	5:6
Chr3	$G^{1:0}$	$V^{0:1}$	$M^{1:2}$	$G^{5:1}$	$M^{1:1}$	$G^{3:0}$	0	$V^{0:9}$	0	G^{1}	$V^{0:2}$	$G^{3:0}$	$V^{0:3}$	$G^{1:0}$	-	$V^{0:143}$	$V^{0:2}$	6:6
Chr4	$M^{2:1}$	$M^{1:2}$	$G^{4:1}$	$G^{10:1}$	$M^{1:1}$	$G^{3:0}$	$G^{1:0}$	$V^{1:7}$	0	?	$V^{0:3}$	$G^{1:0}$	$V^{0:2}$	$V^{0:1}$	-	$V^{0:112}$	$V^{0:1}$	5:6
Chr5	$G^{4:1}$	$V^{0:1}$	$G^{2:0}$	$G^{2:0}$	$V^{0:1}$	$G^{3:0}$	$V^{0:1}$	$V^{2:16}$	0	V^{1}	$V^{0:1}$	$G^{1:0}$	$M^{1:1}$	0	-	$V^{0:153}$	$M^{1:2}$	5:7
G:V	3:0*	0:2*	4:0*	5:0*	1:1	4:0*	2:2	0:5*	0:0	1:1	0:5*	3:1	0:4*	2:1	-	0:5*	0:4*	25:31
reads	11:3	3:5	18:3	25:3	5:4	14:2	2:2	4:47	0:0	1:1	1:13	5:1	1:10	2:1	-	1:625	1:11	94:731

Figure 2 DNA strand age-specific single cell sequencing revealed coordinated but non-directed sister chromatid segregation during pollen meiosis and pollen mitosis 1. In two independent experiments (pollen A1-A9 and pollen B4-B11) we analyzed the segregation of old non-EdU-substituted DNA strands to either the vegetative or generative nucleus in single pollen. Segregation to either the generative (G) or vegetative (V) nucleus was assigned based on the reads ratio G:V displayed in superscript numbers. Segregation for centromeres with reads only from the vegetative or generative sample was assigned to the corresponding cell type (V or G). Segregation for centromeres with reads from both samples was only assigned when one sample contained at least 3x more reads; otherwise it was assigned letter M (mixed). Line 7 represents the assigned segregation ratio G:V for each pollen separately. Ratios pointing to coordinated segregation of old non-EdU-substituted centromeres are marked by an asterisk. Line 8 represents the read ratio G:V for each single pollen across all 5 centromeres. Column 8 represents the segregation ratio G:V for each centromere separately across all 17 pollen. Segregation of all 5 old-non-EdU-substituted centromeres to either the vegetative or generative nucleus is random. Pollen B9 was not

considered because the vegetative sample contained no reads mapping to chloroplast DNA and we might have lost this sample.

Our results reveal, to our knowledge for the first time, coordinated sister chromatid segregation in a metazoan at a genome-wide resolution.

By sequencing premeiotic DNA template strands, DNA strands that served as a template during premeiotic synthesis phase, we can only detect 50% of all meiotic recombination events (Figure 4A-C). Meiotic recombination events where non-EdU-substituted DNA recombines with EdU-substituted DNA (Figure 4B) result in reads assigned to both the vegetative and the generative sample for the same chromosome. However, meiotic recombination events where non-EdU-substituted DNA recombines with non-EdU-substituted DNA (Figure 4C) are not detectable and result in reads assigned only to one of both samples for the same chromosome. In some pollen with coordinated chromatid segregation, we observed the absence of detectable meiotic recombination events (Figure 4D and S7), which implies the dependency of chromatid segregation from the interhomolog choice during meiosis (Figure 4). The increase of significance for coordinated segregation in that pollen and the decrease of significance for coordinated sister chromatid segregation towards both telomeres in all other pollen due to meiotic recombination supports this interpretation of the data (Figure S9A-C). To address and confirm this finding with an alternative method we fluorescently labeled EdU with 6-Carboxyfluorescein by click chemistry[6] and quantified the EdU-6-Carboxyfluorescein fluorescence in both the vegetative and generative nuclei of 133 bicellular pollen (Figure 3A). We distinguished between coordinated sister chromatid segregation with no detectable meiotic recombination events and coordinated sister chromatid segregation with 50% detectable meiotic recombination events by comparing our experimental data with two corresponding models. Based on experimental data about meiotic crossover

positions and frequencies[7] we calculated the theoretical content of EdU in both samples (Figure S11 and S12) in the absence of detectable meiotic recombination events in that 50% of all pollen with coordinated chromatid segregation and 100% detectable meiotic recombination events in that 50% of all pollen with non-coordinated chromatid segregation. For the alternative model, we calculated the theoretical content of EdU in both samples with 50% of detectable meiotic recombination events for both, pollen with coordinated sister chromatid recombination and non-coordinated sister chromatid segregation. By comparing our experimental data with the two models we can confirm the dependency of coordinated sister chromatid segregation from the interhomolog choice during meiosis (Figure 3A). The fact that coordinated sister chromatid segregation only occurs for chromatids, which did not exchange old DNA with newly synthesized DNA is in agreement with the immortal DNA hypothesis. In a control experiment with 12 bicellular pollen (Figure 3B), which incorporated EdU only during pollen mitosis 1, we show that cell fate, either vegetative or generative cell fate, has no influence on the quantified fluorescence signal. We quantified in all 12 pollen the same amount of EdU-6-Carboxyfluorescein fluorescence in both cell types, as expected.

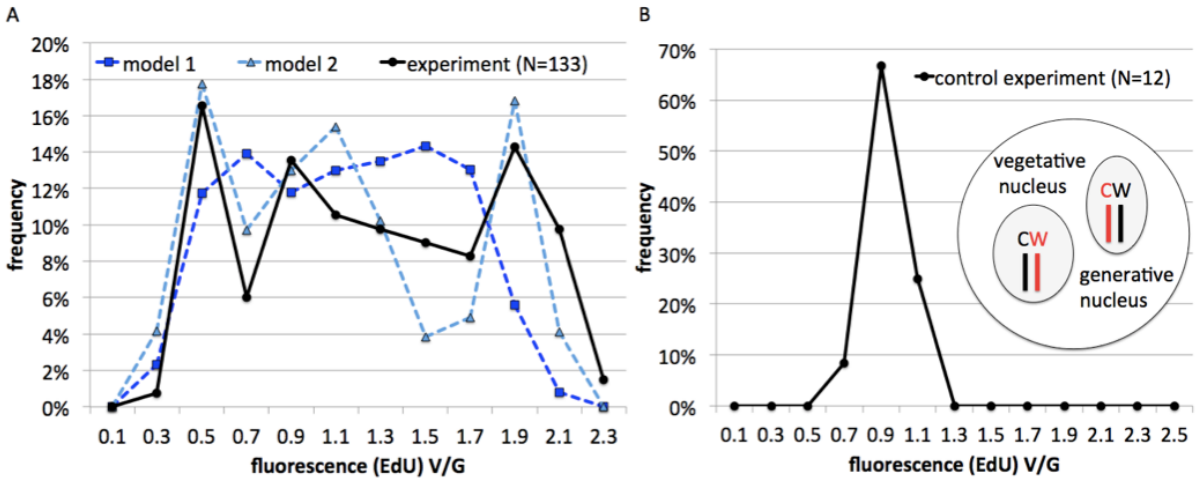

Figure 3 EdU-6-Carboxyfluorescein fluorescence quantification in bicellular pollen confirmed coordinated non-directed sister chromatid segregation dependent from the meiotic interhomolog choice in 50% of all pollen. **A)** To assign segregation of old non-EdU-substituted DNA strands to either the vegetative or generative nucleus we

quantified the amount of EdU-6-Carboxyfluorescein fluorescence in the vegetative and generative nuclei of bicellular pollen N = 133; 63 Col-0 and 70 L*er*; see Figure S8A), which incorporated EdU before meiosis and mitosis 1. The coordinated segregation of all 5 non-EdU-substituted DNA strands would result in a vegetative to generative nuleus (V/G) ratio of 2:0 or 1:2 depending on the direction of coordinated segregation. Non-directed coordinated segregation to either the vegetative or generative nucleus would result in two peaks, one at a V/G ratio of 0.5 and one of 2.0. V/G ratios between 0.5 and 2.0 point to non-coordinated segregation of the 5 non-EdU-substituted DNA strands or might be caused by meiotic recombination (Figure 4). To test the dependency of coordinated sister chromatid segregation from the meiotic interhomolog choice we calculated 2 models based on experimental data about the location and frequency of meiotic crossovers. Model 1 represents the expected frequency distribution for coordinated non-directed sister chromatid segregation independent from the interhomolog choice during meiosis. Model 2, which fits best our experimental data, represents the expected frequency distribution for coordinated non-directed sister chromatid segregation dependent from the interhomolog choice during meiosis. **B)** Control experiment (N = 12): EdU incorporation during S-phase before mitosis 1 only. The vegetative and the generative nuclei contain the same amount of EdU-6-Carboxyfluorescein fluorescence, and thus the two cell types have no impact on the quantified amount of EdU-6-Carboxyfluorescein.

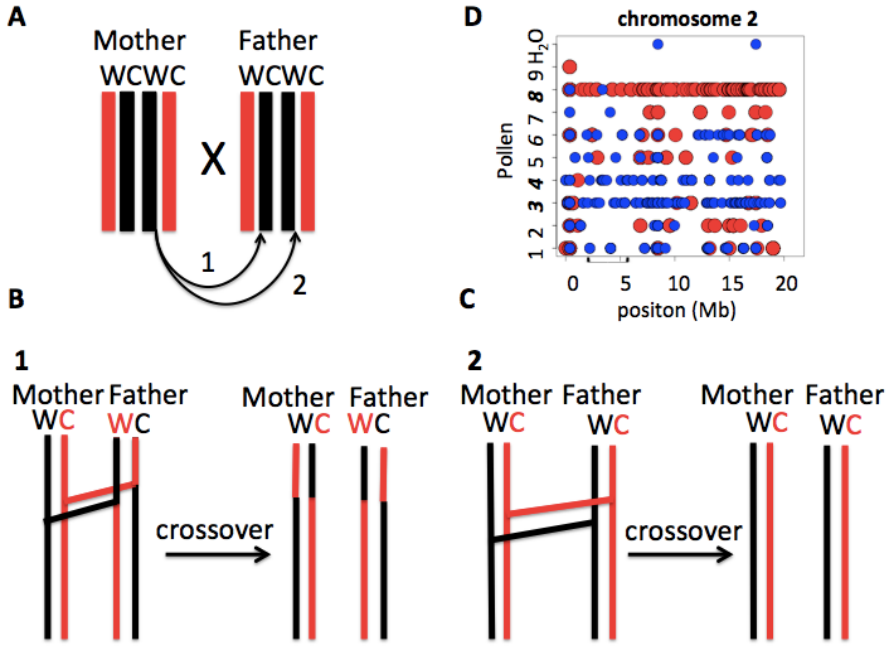

Figure 4 Coordinated sister chromatid segregation depends on the meiotic interhomolog choice. **A)** During meiotic recombination a chromatid from the chromosome inherited from the mother may recombine with one of both chromatids of the chromosome inherited from the father (choice 1 or 2), assuming a strong interhomolog bias. Depending on the interhomolog choice there will be an exchange of DNA of different ages or of EdU-subsituted with non-EdU-substituted DNA. **B)** Choice 1 results in an exchange of DNA of different ages. The old template Watson (W) strand recombines with the newly synthesized Watson strand from the other chromosome. **C)** Choice 2 results in no exchange of DNA of different ages. The old Watson strand recombines with the old Watson strand of the other chromosome. **D)** Example data for

chromosome 2 that indicates no visible meiotic recombination in pollen A3, A4, and A8 (bold). Blue spots represent reads from the generative sample, and red dots represent reads from the vegetative sample.

With a new method, allowing to track the segregation of each sister chromatid separately for the first time in metazoans, we confirm the existence of coordinated sister chromatid segregation during *Arabidopsis thaliana* pollen development. Coordinated sister chromatid segregation is one of two essential conditions to accept the immortal strand hypothesis. Together with its dependency from the interhomolog choice during meiotic recombination, this mode of sister chromatid segregation leads to the prevention of DNA replication errors in one of two daughter cells (Figure S10). However, the choice of the daughter cell was random. Sister chromatid segregation was not directed, and all 5 non-EdU-substituted DNA strands randomly segregated together either into the vegetative or the generative nucleus.

Our results find the existence of coordinated sister chromatid segregation in plants and support the immortal strand hypothesis. However, the hypothesis can't be applied to pollen and thereby to male gamete development due to the absence of directed chromatid segregation. The existence of a mechanism for coordinated sister chromatid segregation yet points to the existence of directed sister chromatid segregation in other plant tissues, for example in meristematic stem cells.

Limited evidence in the literature supports our finding of coordinated sister chromatid segregation at a single chromatid resolution. Yadlapalli and Yamashita (2013)[8] published data pointing to the coordinated segregation of Watson or Crick strands of chromosome homologs, referred as WW::CC segregation, in *Drosophila* autosomes (comment Sauer and Klar). Armakolas and Klar 2006[9] reported the coordinated segregation of Watson or Crick strands of chromosome 7 homologs in mouse embryonic stem cells. Various studies reported the existence of coordinated sister chromatid

segregation by looking at total chromatid segregation (reviewed in Yadlapalli and Yamashita 2013)[5].

Methods Summary

Current methods rely on the incorporation of 5-Brom-2-desoxyuridin (BrdU) to label newly synthesized DNA strands. Old template DNA strands are distinguished from newly synthesized BrdU-substituted strands by digesting BrdU-substituted strands with ExonucleaseIII upon UV-photolysis[10]. The identity of the undigested template strand, either Watson 5'→3' or Crick 3'→5', is determined by probe hybridization.

To monitor label incorporation in tissues, we substituted BrdU for 5-Ethynyl-2'-deoxyuridine (EdU). Photolysis is replaced by an uracil N-glycosylase enzymatic reaction and the segregation of the only non-EdU-substituted DNA strands, either to the vegetative or to one of both sperm nuclei of *Arabidopsis thaliana* pollen is determined by whole genome sequencing upon whole genome amplification (Figures S1-S5).

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Methods

Plant Material Experiments were performed using *Arabidopsis thaliana* pollen from either a marker line for sperm nuclei (*Duo1:H2B-mRFP* ; Col-0 accession; pollen A1-A9) or from a wild-type line (L*er* accession; pollen B4-B11). The marker line is a donation from David Twell. The marker or accession did not influence our results (Figure S9).

EdU incorporation and detection in *Arabidopsis thaliana* pollen. Cut inflorescences from young *Arabidopsis thaliana* plants were grown in liquid MS medium (4.3g/L Murashige and Skoog salt (Carolina Biological Supply Company, Burlington, North Carolina, USA), 10g/L sucrose (Applichem GmbH, Darmstadt, Germany), pH5.6) containing 100uM EdU (5-Eghynyl-2'-deoxyuridine (Sigma-Aldrich , Buchs, Switzerland #T511285) and 100ug/mL Ampicillin. Open flowers were removed every 24h. Anthers from open flowers were dissected and destroyed in water to collect mature pollen. The

pollen suspension was centrifuged and only 20uL water was left in the tube. To fluorescently label EdU by a click-reaction we followed the method of Kliszczak *et al.* 2011[6] and added in the following order: 1uL of 0.1M 6-Carboxyfluorescein-TEG azide (Berry & Associates Inc., Dexter, USA # FF6110), 10uL of 6mM Copper(II)sulfate (Sigma-Aldrich , Buchs, Switzerland) and 15uL of 30mM (+)-Sodium-L-ascorbate (Sigma-Aldrich , Buchs, Switzerland). After 30min incubation at room temperature, the pollen suspension was washed 3x with 200uL water by centrifugation and discarding the supernatant. The final pollen suspension was stored at -80°C in 50uL water and 50uL DAPI solution A (0.1M sodium phosphate, 1mM EDTA, 0.1% Triton-X-100, 0.4ug DAPI) for EdU-6-Carboxyfluorescein fluorescence quantification or DAPI solution B (0.4ug DAPI/10mL H_2O) for chromatid age-specific single cell sequencing. Both, EdU-6-Carboxyfluorescein fluorescence quantification and chromatid age-specific single cell sequencing was done in two independent experiments; pollen A1-A9 and pollen B4-B11.

EdU-6-Carboxyfluorescein fluorescence quantification in *Arabidopsis thaliana* bicellular pollen. To quantify the amount of EdU-6-Carboxyfluorescein in the vegetative and generative nucleus of bicellular pollen, cut inflorescences were grown for 72-96 hours in 100uM EdU containing MS medium. To test for EdU incorporation during both pollen meiosis and pollen mitosis 1, anthers of different flower buds from the same inflorescence were analyzed separately. For EdU incorporation during pollen meiosis and pollen mitosis 1 the following pattern should be observed: oldest flower bud containing bicellular pollen: contains a mixture of tricellular pollen (from the longer filament) and bicellular pollen (from the shorter filament) and all nuclei contain EdU-6-Carboxyfluorescein ; next 1-3 younger flower buds contain only bicellular pollen with EdU-6-Carboxyfluorescein in both the vegetative and generative nuclei ; next 1-2 flower buds contain a mixture of bicellular and monocellular pollen with EdU-6-

Carboxyfluorescein in both nuclei. These are the pollen to select for EdU-6-Carboxyfluorescein fluorescence quantification. For the control experiment with EdU incorporation during mitosis 1 only, inflorescences were grown 48 hours in 100uM EdU containing MS medium. For each pollen grain, Z-stack data acquired on the Leica DM-6000 epifluorescence microscope (Leica, Wetzlar, Germany) equipped with an sCMOS camera (ANDOR, UK, Neo sCMOS DC152Q-FI) was compressed into a single plane using the SUM Slice function in ImageJ. Individual nuclei were selected using the freeform drawing tool to produce a region of interest (ROI). By using the 'Measure' function the area, the mean gray value and integrated density of the ROI were measured. The mean background was measured by repeating the same procedure for the non-fluorescent region around the nucleus. Finally, the corrected total nucleus fluorescence (CTNF) was calculated using the formula: CTNF = Integrated density of nucleus ROI – (Area of ROI x mean fluorescence of background).

Strand segregation model calculation. The computer-aided design of two models for two different scenarios of DNA strand segregation is based on published data containing meiotic crossover frequencies during male meiosis of *Arabidopsis thaliana* from Giraut *et al.* 2011[7]. Depending on the location of the meiotic crossover one chromatid loses (case A) a certain amount of newly synthesized EdU-substituted DNA whereas the other chromatid gets some additional (case B) EdU-substituted DNA. One of both chromatids segregates into the vegetative and one into the generative nucleus. One can calculate two possible V/G ratios; caseA:caseB and caseB:caseA for each crossover. By taking into account the frequency of each crossover, the frequency of each possible V/G ratio in a certain ratio window can be calculated. We used a ratio window of 0.2. To calculate the frequency for a certain V/G ratio over all 5 chromosomes, an AWK script was applied to compute all possible combinations. To consider the different chromosome lengths and

for that reason the different contribution of each calculated V/G ratio from each chromosome we multiplied, before summing up all 5 V/G ratios from one possible combination, each V/G ratio by the percentage of the corresponding chromosome from total chromosomal DNA. The corresponding frequencies for all 5 V/G ratios from one possible combination were multiplied (Figure S11).

Pollen lysis. Pollen has to be lysed for laser capture microdissection in a way that the 3 nuclei are not separated but distant enough to separate them (Figure S2C). Laser capture microdissection on intact pollen did not work because the laser can't cut the pollen wall and the 3 nuclei are too close. A pollen suspension was centrifuged at maximum speed for 2min. Supernatant was removed and replaced with 100uL sperm extraction buffer (SEB)[11]. The pollen suspension was then transferred into a 2mL tube containing 2 glass beads of 5-7mm in diameter (Sigma-Aldrich, Buchs, Switzerland, solid glass beads diam. 5mm, Z265942-1EA) (correct glass bead size is critical!). After vortexing for 30s, 10uL DAPI solution B were (0.4ug DAPI/10mL H_2O) added and the glass beads removed by a forceps. The pollen suspension was transferred into a 0.2mL PCR tube and centrifuged at maximum speed for 2min. The supernatant was removed and replaced by water. This step was repeated to remove salts from the pollen suspension. Salts disturb the laser during laser capture microdissection.

Isolation of vegetative and sperm nuclei from single pollen by laser capture microdissection. 1uL droplets of the pollen suspension were pipetted onto PE membrane-mounted metal frame slides and analyzed for the presence of non-separated nuclei from single lysed pollen under an epifluorescence microscope (Leica DM6000). Pictures were taken to remember the position of the nuclei. Slides were air dried at room temperature for about 1 hour and sprayed with conventional hair spray (Migros I

am Ultra Strong) to fix the nuclei on the membrane. Slides were again air dried for about 1 hour. Microdissection was performed with an MMI CellCut Plus device (MMI Molecular Machines & Industries AG, Glattbrugg, Switzerland). Two samples per pollen were collected; one sample containing the vegetative nucleus and one sample containing both sperm nuclei (Figure S2C). Isolated nuclei were collected using MMI isolation caps and stored at -80°C. All isolated samples were checked for the presence of the isolated nuclei under an epifluorescence microscope. For the first experiment's water control (pollen A1-A9) a region without DAPI or 6-Carboxyfluorescein fluorescence was cut. No laser capture microdissection was performed for the second experiment's water control, which was generated during whole genome amplification.

In-vitro Assay to test the specificity of recombinant *E. coli* G/U mismatch-specific DNA glycosylase (MUG) for EdU and EdU-6-Carboxyfluorescein. Liu *et al.* 2002[12] reported the specificity of MUG for various uracil 5-substituents, but not for EdU. We repeated their assay (Figure S4) by ordering the same oligonucleotides (5'-GGCTATCGTGGCXGGCCACGACGG-3', 5'-GGCTATCGTGGCTGGCCACGACGG-3' and 3'-GAGTCCGATAGCACCGACCGGTGC-5') (Microsynth AG, Balgach, Switzerland) for X = EdU. Since we labeled EdU with 6-Carboxyfluorescein with click chemistry, we also tested the specificity of MUG for EdU-6-Carboxyfluorescein (Figure S4D). Hybridized oligonucleotides were incubated in a click reaction cocktail as described above and purified by ethanol precipitation. Reaction samples were analyzed on a 24% polyacrylamide gel by silver staining[13].

In-vitro Assay to test PCR inhibition by EdU-6-Carboxyfluorescein. According to linktech product guide 2010 (Link Technologies Ltd.), 6-Carboxyfluorescein allows the effective blockage of the 3'-terminus from polymerase extension. In addition, we could

not find a protocol for PCR amplification of click-reaction modified DNA. We assessed the compatibility of PCR with EdU containing DNA before and after click-reaction with 6-Carboxyfluorescein-TEG azide in vitro. In a first PCR reaction, EdU containing DNA was synthesized by using 0.8uL of a 10mM dNTP mix containing EdUTP (5-Ethynyl-dUTP, Jena Bioscience, CLK-T07) and 0.2uL of a 10mM dNTP mix containing dTTP. The same PCR was done with 1uL of 10mM dNTP mix with dTTP as a control. The PCR product was run on an agarose gel and bands were cut and column purified. Click reaction was performed by mixing 30uL column purified DNA with 1uL 0.1M 6-Carboxyfluorescein-TEG azide, 30uL 6mM Copper(II)sulfate and 30uL 30mM (+)-Sodium-L-ascorbate. The mixture was incubated for 25min in the dark and column purified. DNA concentration was measured using a Nanodrop (Thermo Fisher Scientific Inc.) and all samples were adjusted to the same concentration by dilution with water. PCR reaction was repeated with EdU substituted DNA without click-reaction and with click-reaction with 6-Carboxyfluorescein-TEG azide and without any azide compound and with non-EdU substituted DNA. PCR products were loaded and analyzed on an agarose gel (Figure S5).

Nucleus lysis, EdU-specific DNA strand digestion and whole genome amplification. Nuclei were directly lysed on the isolation caps by following the New England Biolabs PicoPLEX WGA Kit (New England Biolabs #E2620S) Pre-Amplification Protocol from step 1 to step 3. 5uL of cell extraction buffer and 5uL of extraction cocktail were pipetted into the isolation tube and by inverting the tube placed on the isolation cap. Parafilm wrapped caps were incubated in water baths according to step 3 of the protocol. Lysed nuclei were centrifuged and 1uL of recombinant *E. coli* G/U Mismatch-Specific DNA Glycosylase (MUG) (Novoprotein #C152) was added and incubated for 10 minutes at 37°C. Next, 1uL of E. coli Exonuclease III (New England Biolabs #0206) was added and incubated for 5 minutes at 37°C. After heat inactivation for 10 minutes at 70°C, the

PicoPlex WGA Kit Protocol was continued with step 4 of the PreAmplification protocol. Finally, the products of whole genome amplification were purified by a silica column (Macherey Nagel #740609.50 NucleoSpin Gel and PCR Clean-up kit). Before trashing the laser capture microdissection isolation caps, they were checked for the disappearance of any DAPI or/and EdU-6-Carboxyfluorescein fluorescence to check for successful nucleus lysis.

Illumina HiSeq 2500 library preparation. Barcoded libraries were prepared with the Ovation Ultralow DR Multiplex System 1-96 (NUGEN #0329). Libraries were prepared with 100ng of column purified whole genome amplification product. Library preparation was done following the kit's protocol except for the magnetic bead purification steps. Agencourt AMPure XP magnetic beads (Beckman Coulter #A63881) were used according to the product's protocol; 1.8uL AMPure XP beads per 1.0uL of the sample. Except for the second experiment's water control, an equimolar amount of each library was pooled into a single library and sequenced on the Illumina HiSeq2500 platform (Functional Genomics Center, University of Zürich).

Sequencing data analysis. Raw sequencing data was converted by using FASTQ Groomer[14] with basic options. Converted files were mapped to the human genome (Homo sapiens hg19) genome with Bowtie2[15,16] with the very sensitive local option to remove contaminating human reads. SAMtools were used to filter and keep only reads, which were not mapped and to convert into a Sam file. Picard tools were used to convert from Sam to Fastq. Fastq files were mapped to the *Arabidopsis thaliana* TAIR10 genome with Bowtie2 with the fast local option. SAMtools were used to filter for reads with a minimum mapping quality of 30. Mapped reads were sorted with an AWK script and

visualized in R. Centromere regions were defined according to Hosouchi *et al.* 2002[17] +/- 1 Mb.

Supplementary Information

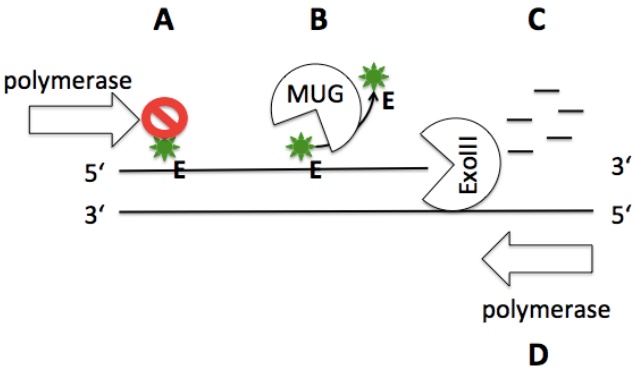


Figure S1 EdU-specific strand digestion. Our assay to distinguish between the old template DNA strand and the newly synthesized DNA strand is based on the incorporation of EdU during S-phase before meiosis or mitosis. To visualize the incorporation of EdU we labeled EdU with 6-Carboxyfluorescein by a chemical click reaction. EdU-6-Carboxyfluorescein blocks the PCR reaction (A) and whole genome amplification. For that reason EdU-6-Carboxyfluorescein is removed by E. coli MUG (B), an uracil N-glycosylase, leaving an abasic site (Figure S4). This abasic site is recognized by ExonucleaseIII (C), which digests the EdU-6-Carboxyfluorescein containing strand 3'→5' (Figure S4). The old DNA template strand containing no EdU can be amplified by whole genome amplification (D).

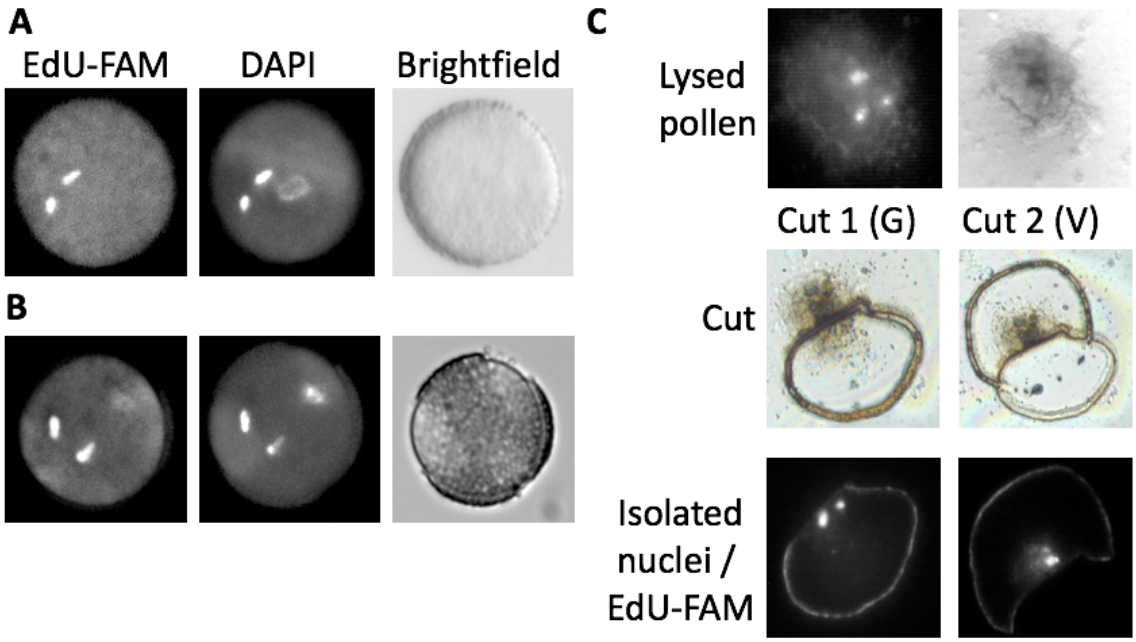


Figure S2 EdU incorporation during pollen development and nuclei isolation by laser capture microdissection. EdU-FAM = EdU-6-Carboxyfluorescein **A)** EdU incorporation into pollen during S-phase before pollen mitosis 2. **B)** EdU incorporation into pollen

during S-phases before mitosis 1, mitosis 2 and maybe meiosis. **C)** pollen lysis in a way that the 3 nuclei stay together and laser capture microdissection of the two generative and the vegetative nuclei.

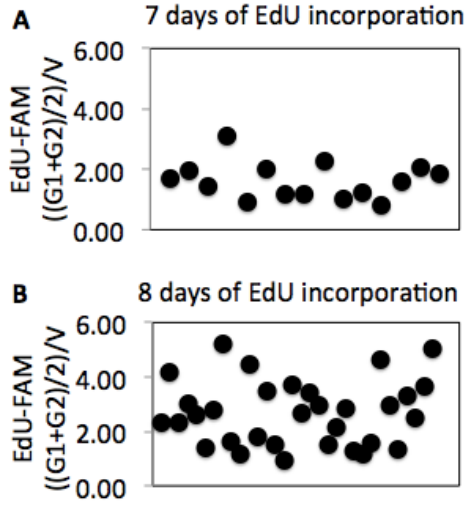


Figure S3 Increase in the variation of EdU distribution after incorporation during meiosis. To make sure that EdU has been incorporated during meiosis we quantified EdU in the vegetative and the generative nuclei after 7 and 8 days of EdU incorporation. By looking at the ratio of EdU-6-Carboxyfluorescein in generative (G)/vegetative (V) nuclei we observed an increase in the variance after 8 days, which can be explained by meiotic recombination.

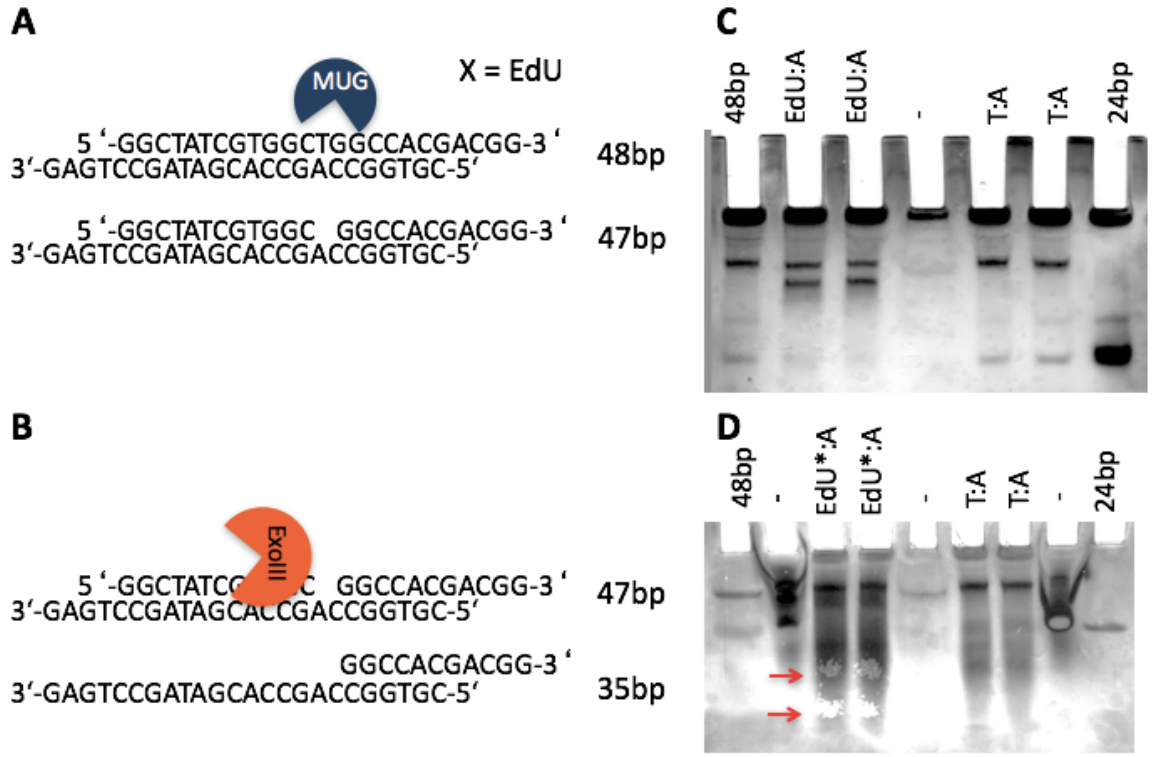


Figure S4 EdU- and EdU-6-Carboxyfluorescein specific strand digestion by *E. coli* MUG and Exonuclease III. **A-C)** EdU-specific strand digestion by *E. coli* MUG and Exonuclease III. MUG, an uracil-N-glycosylase, recognizes EdU and produces an abasic site, which is recognized by Exonuclease III. To test the specificity of MUG we digested

short DNA oligomers with either EdU or Thymidine at a particular position. If EdU is recognized by MUG **(A)**, an abasic site is produced, which is recognized by Exonuclease III **(B)**. Exonuclease III digests the strand 3'→5'. Separation of the digestion products on a polyacrylamide gel and silver staining reveals that MUG specifically recognizes EdU **(C)** and EdU-6-Carboxyfluorescein **(D)** but not Thymidine (C and D). **D)** EdU-6-Carboxyfluorescein specific strand digestion by *E. coli* MUG and Exonuclease III. Overlay of silver stained gel with fluorescence picture (6-Carboxyfluoresceine fluorescence, red arrows)

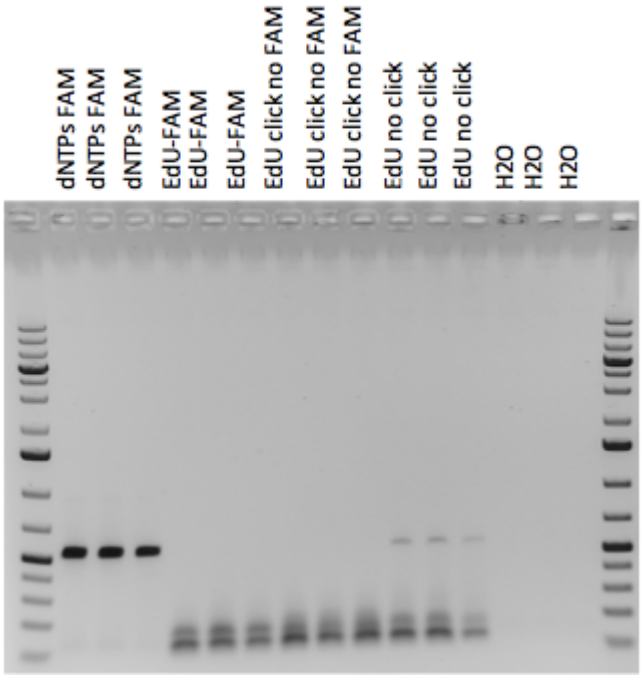


Figure S5 PCR inhibition by EdU. To test whether EdU, EdU-6-Carboxyfluorescein (EdU-FAM) or the chemical click reaction has an influence on PCR, we synthesized EdU substituted DNA. In a first step, a random PCR reaction (540bp) with a 5-Ethynyl-dUTP containing dNTP mix was done to produce EdU substituted PCR products. This PCR product was either coupled with 6-Carboxyfluorescein by a click reaction (EdU-FAM) or incubated with all click reaction compounds except 6-Carboxyfluorescein-TEG azide (EdU click no FAM) or not modified at all (EdU no click). The DNA content of all products was quantified with Nanodrop, and all products were diluted to the same DNA concentration. The diluted products were again amplified in a second PCR. As a control, the same PCR was done with a standard non 5-Ethynyl-dUTP containing dNTP mix, incubated with the click reaction compounds (dNTPs FAM), diluted to the same concentration as the EdU-containing products and amplified in a second PCR. All EdU containing template DNA incubated with the click reaction reagents could not be amplified after click reaction. The amplification of EdU containing DNA, which was not incubated with the click reaction compounds is weaker compared to the amplification of normal non-EdU containing DNA.

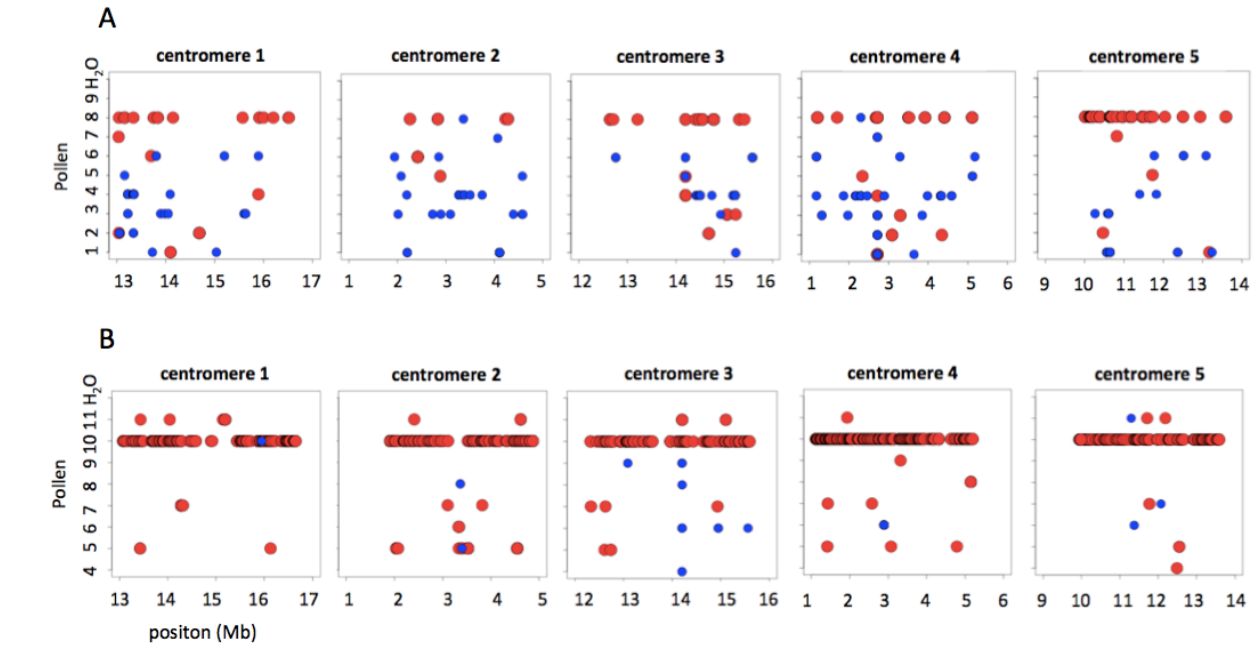


Figure S6 Chromatid-age specific single cell sequencing results for reads mapped to the centromere region. **A)** Y-axis: pollen A1-A9 and water control. X-axis: mapped position on the centromere +/- 1Mb. Red spot: read from the vegetative sample. Blue spot: read from the generative sample. **B)** Repetition of the experiment with pollen B4-B11.

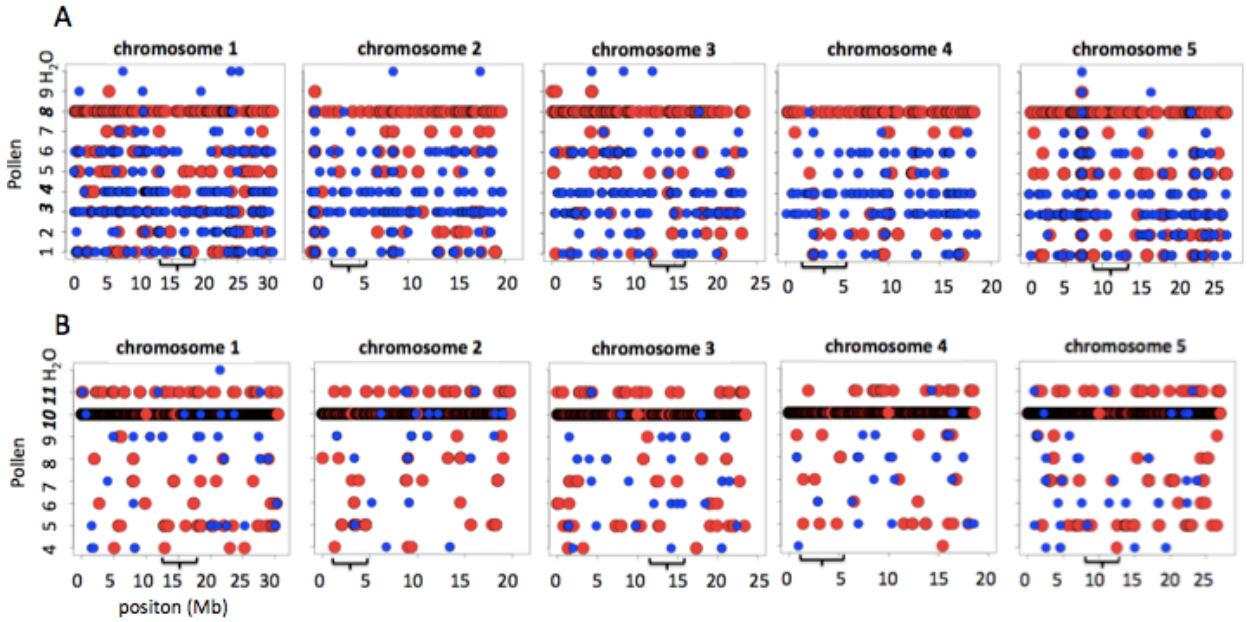


Figure S7 Chromatid-age specific single cell sequencing results for reads mapped to the *Arabidopsis thaliana* genome. Pollen numbers in bold: No visible meiotic recombination. **A)** Y-axis: pollen A1-A9 and water control. X-axis: mapped position on the chromosome. Red spot: read from the vegetative sample. Blue spot: read from the generative sample. **B)** Repetition of the experiment with pollen B4-B11.

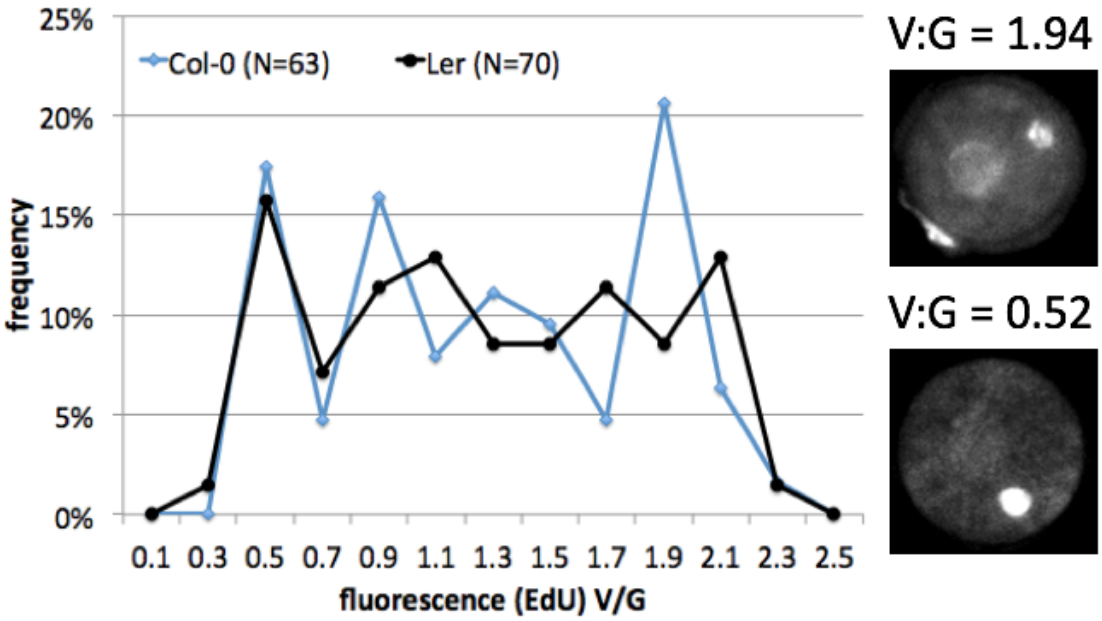


Figure S8 *Arabidopsis thaliana* accession and *Duo1:H2B-mRFP* marker does not affect strand segregation. For the development of the age-specific DNA strand sequencing assay, we used pollen from a *Duo1:H2B-mRFP* marker line of the Col-0 accession (pollen A1-A9) to distinguish between sperm and vegetative nuclei. However, the size of the nuclei and also often the proximity of the nuclei allows dstinghishing between the nuclei in wild-type. For that reason, we repeated the experiment with pollen from a wild-type line of the L*er* accession (pollen B4-B11). Also for EdU-6-Carboxyfluorescein quantification in bicellular pollen, we used both lines. By comparing both results, there is no difference between both plant lines. The peak at a V/G ratio of 1.9 is flattened in the L*er* accession compared to the Col-0 accession. However, this is due to the 0.2 window size, and by merging the peaks at a ratio of 1.9 and 2.1 the result looks similar. Next to the diagram are two example pictures of bicellular pollen quantified for their content of EdU-6-Carboxyfluorescein fluorescence. The pictures show one single plane from a Z-stack. The upper pollen was quantified to have a V:G ratio of 1.94 whereas the pollen below was quantified to have a V:G ratio of 0.52.

A left telomere

pollen	reads in cell with more reads	expected if random	total reads	P for random segregation
A1	18	13.5	27	0.035
A2	6	5	10	0.205
A3	54	31	62	7.33E-10
A4	31	18	36	5.49E-06
A5	9	7	14	0.122
A6	15	13	26	0.115
A7	5	4.5	9	0.246
A8	67	35	67	6.78E-21
B4	5	4.5	9	0.246
B5	10	7	14	0.061
B6	5	3	6	0.094
B7	10	6.5	13	0.035
B8	5	4.5	9	0.246
B10	1196	599	1198	0.00E+00
B11	21	11.5	23	3.02E-05

B centromere

pollen	reads in cell with more reads	expected if random	total reads	P for random segregation
A1	11	7	14	**0.022**
A2	5	4	8	0.219
A3	18	11	21	**0.001**
A4	25	14	28	**1.22E-05**
A5	5	5	9	0.246
A6	14	8	16	**0.002**
A7	2	2	4	0.375
A8	47	26	51	**1.11E-10**
B4	1	1	2	0.500
B5	13	7	14	**0.001**
B6	5	3	6	0.094
B7	10	6	11	**0.005**
B8	2	1	2	0.250
B10	625	313	626	**2.25E-186**
B11	11	6	12	**0.003**

C right telomere

pollen	reads in cell with more reads	expected if random	total reads	P for random segregation
A1	16	12	24	0.044
A2	13	12	24	0.149
A3	44	26.5	53	4.92E-07
A4	36	19	38	2.56E-09
A5	8	6.5	13	0.157
A6	22	16	32	0.015
A7	5	4.5	9	0.246
A8	68	34.5	69	1.17E-19
B4	1	0.5	1	0.500
B5	19	11.5	23	**0.001**
B6	5	3	6	0.094
B7	6	5.5	11	0.226
B8	3	3	6	0.313
B10	1323	663	1324	0.00E+00
B11	30	16.5	33	6.35E-07

Figure S9 Statistics for chromatid cosegregation at **A)** left telomere, **B)** centromere, **C)** right telomere. Pollen in bold letters/numbers: no visible recombination expected (interpreted from Figure S7). P values in bold: significant chromatid cosegregation at the

centromere (exact test for goodness-of-fit: binomial test). P values in red: decreased significance compared to the centromere. P values in green: increased significance compared to the centromere. Green shaded: significant chromatid cosegregation. Interpretation: significance increases toward the telomere in all 5 pollen, which show no visible recombination events. This is due to a higher number of reads at the telomeres. All other pollen show a decrease of significance towards the telomeres due to meiotic recombination.
This data supports the interpretation that no visible meiotic recombination occurred in pollen A3, A4, A8, B10, and B11. 9 pollen are significant for chromatid cosegregation at the centromere, 7 at the left telomere and 8 at the right telomere.

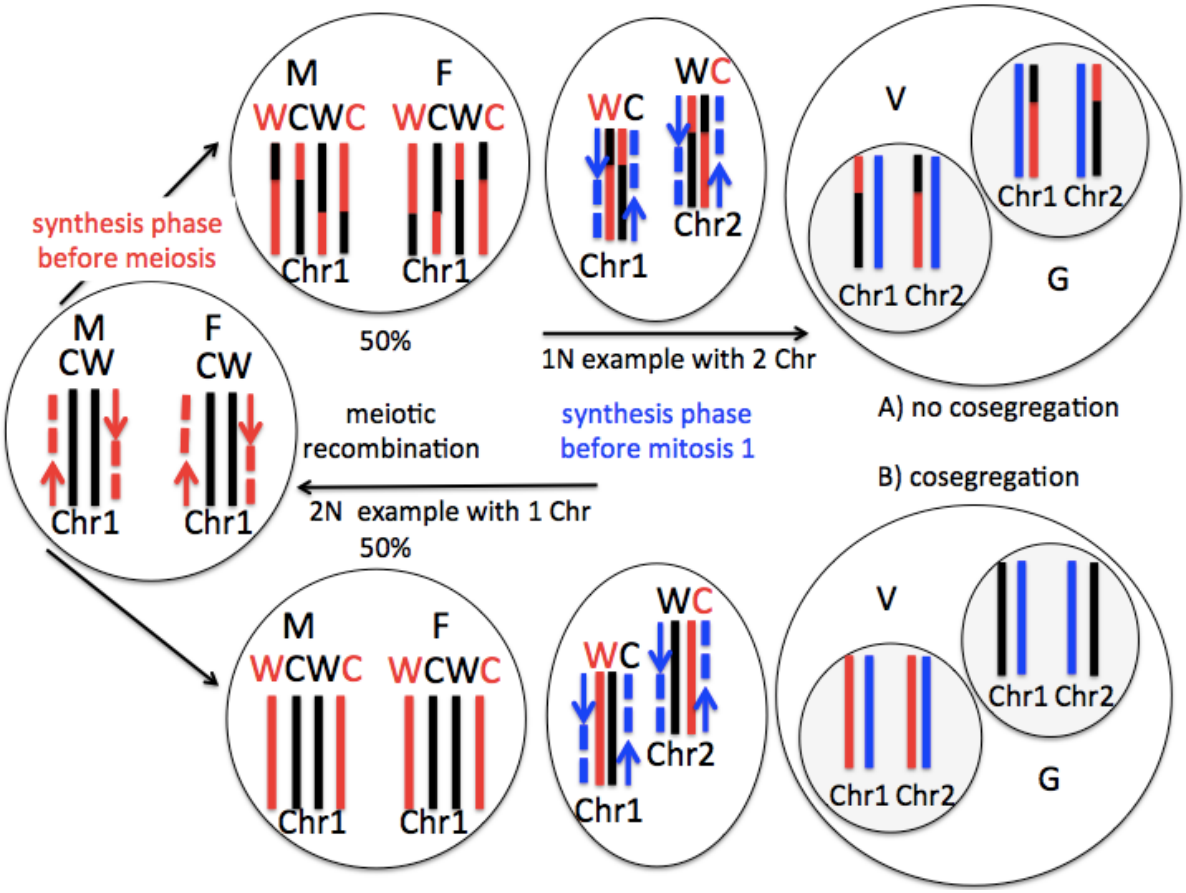


Figure S10 Working model Upper pathway: 50% of all microspores only inherit chromatids, which recombined old template strands (black) with strands newly synthesized during premeiotic S-phase (red). No chromatid age-dependent sister chromatid segregation may be observed in this pollen. Both nuclei inherit chromatids containing DNA strands partly synthesized during premeiotic S-phase (red) or S-phase before mitosis 1 (blue). Lower pathway: 50% of all microspores only inherit chromatids, which did not recombine old template strands with newly synthesized strands during premeiotic S-phase. Chromatids cosegregate in a chromatid age-dependent manner during mitosis 1. One of both nuclei inherits only chromatids containing the premeiotic DNA template strand, which was present already before premeiotic S-phase.

A	EdU content for recombination events in front of the centromere possibility A)	("chromosome length in bp" x "mean proportion AT along chromosome") - (("stop position of recombined region" x "mean proportion AT along recombined region")/2)
B	EdU content for recombination events in front of the centromere possibility B)	(("chromosome length in bp" x "mean proportion AT along chromosome")/2) + ("stop position of recombined region" x "mean proportion AT along recombined region")
C	EdU content for recombination events after the centromere possibility A)	("chromosome length in bp" x "mean proportion AT along chromosome") - ((("chromosome length in bp" - "start position of recombined region") x "mean proportion of AT along recombined region")/2)
D	EdU content for recombination events after the centromere possibility B)	(("chromosome length in bp" x "mean proportion AT along chromosome")/2) + (("chromosome length in bp" - "start position of recombined region") x "mean proportion of AT along recombined region")
E	EdU ratio V/G possibility A	A/B or C/D
F	EdU ratio V/G possibility B	B/A or D/C
G	frequency for a certain EdU ratio V/G window for 1 chromosome	$\sum crossover\_frequencies$
H	Calculation of 1 possibility for an EdU ratio from 2 chromosomes	("EdU ratio chromosome1" x "proportion chromosome1 from total chromosomal DNA") + (EdU ratio chromosome2 x "proportion chromosome2 from total chromosomal DNA")
K	frequency for calculated EdU ratio in H	"cross over frequency chromosome 1" x "crossover frequency chromosome2"

Figure S11 Calculation of the models for the EdU content in vegetative and generative nuclei. To consider the impact of meiotic recombination on the distribution of EdU between vegetative and generative nuclei of pollen, we first calculated the theoretical content of EdU in one cell by applying formula A-D. From these values we calculated the ratio of EdU in the vegetative (V) against EdU in the generative (G) cell (E and F). To calculate the frequency of a certain EdU ratio for a single chromosome (e.g. ratio 0.5-0.7) we summed all the corresponding crossover frequencies from meiotic crossovers, which resulted in a V/G ratio between 0.5-0.7 (G). To calculate the possible EdU ratios from all 5 chromosomes combined, we first multiplied the single EdU ratio values (A-D) by the DNA proportion of each corresponding chromosome from total chromosomal DNA. With an AWK script, we computed all possible crossover combinations for all 5 chromosomes and summed up the corresponding EdU ratios (H). To calculate the frequency of each possible combination we multiplied the corresponding crossover frequencies (K).

```
for a in $(awk '{print $1}' chr12345.txt)
do
  for b in $(awk '{print $2}' chr12345.txt)
  do
    echo $a $b
  done
done > allpossiblecombinations.txt
```

Figure S12 Basic script to compute all possible combinations of EdU ratios from two different chromosomes. Column 1 from file chr12345.txt contains all possible EdU ratios from one chromosome and column 2 from the same file contains all possible EdU ratios from the other chromosome. The script prints all possible combinations between column 1 and 2 into file allpossiblecombinations.txt.

Chapter 4 – Introduction 2

WYR: From Asymmetry to Haploinsufficiency

Non-random sister chromatid segregation (Chapter 1-3) must involve asymmetric proteins, which confer the non-random segregation of sister chromatids to both daughter cells.

In budding yeast both the kinetochore protein Ndc10[1] and Sli15 (Chapter 5), which is required for Ndc10 recruitment, segregate asymmetrically during mitosis. In plant a mutation in *WYR*, the ortholog of *SLI15* has been described to cause cell fate decision defects during female and male *Arabidopsis thaliana* gametogenesis[2]. We supposed that WYR might be asymmetrically distributed as well and that this asymmetric distribution might impact cell fate decisions through the asymmetric or non-random segregation of sister chromatids (Chapter 1-3).

However, I could show that directed sister chromatid segregation during male gametogenesis does not exist (Chapter 3) and hence WYR is not supposed to be involved in directed non-random sister chromatid segregation during male gametogenesis. Mouse[3] *Incenp* and fly[4] *incenp* mutant alleles, the orthologs of *WYR*, cause chromosome segregation defects and aneuploidy during female meiosis. In both, mouse and plant, heterozygous *wyr*- or *Incenp* disrupted progeny is phenotypically not distinguishable from their wild-type littermates. Selfing or intercrossing the heterozygotes results in no live-born homozygous progeny[3]. Based on the similar phenotype of *wyr-1* in plant and *Incenp* in mouse I came up with the hypothesis that the observed phenotypes of *wyr-1* are caused by aneuploidy. Experiments and results are described in Chapter 5.

References

1. Thorpe, P. H., Bruno, J. & Rothstein, R. Kinetochore asymmetry defines a single yeast lineage. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 6673–6678 (2009).
2. Kirioukhova, O. *et al.* Female gametophytic cell specification and seed development require the function of the putative Arabidopsis INCENP ortholog WYRD. *Development* **138,** 3409–3420 (2011).
3. Cutts, S. M. *et al.* Defective chromosome segregation, microtubule bundling and nuclear bridging in inner centromere protein gene (Incenp)-disrupted mice. *Hum. Mol. Genet.* **8,** 1145–1155 (1999).
4. Resnick, T. D. *et al.* Mutations in the chromosomal passenger complex and the condensin complex differentially affect synaptonemal complex disassembly and metaphase I configuration in drosophila female meiosis. *Genetics* **181,** 875–887 (2009).

Chapter 5 – Manuscript 2

Introduction

WYR[1] has first been described in a mutation screen for impaired egg cell differentiation in *Arabidopsis thaliana.* Mutations in *WYR* lead to the formation of two egg cells at the expense of accessory synergids, impaired mitotic divisions in the male gametophyte and have a parental effect on embryo cytokinesis. *WYR* encodes a putative plant ortholog of the human inner centromere protein (*INCENP*) and yeast *SLI15*. INCENP is a regulatory component of the chromosome passenger complex (CPC) that also includes AURORA-B kinase, SURVIVIN, and BOREALIN. All members of CPC belong to the passenger centromere-interacting proteins, which other than the constitutively binding centromere-interacting proteins localize to the centromere only during specific stages of the cell cycle. CPC plays a role in the correction of kinetochore-microtubule attachments. At the centromere Bir1 together with Sli15 mediate a linkage between the centromere and microtubules. This linkage is required for the correction of syntely presumably by measuring the kinetochore tension during mitotic chromosome segregation[2].

As in plants, heterozygous *Incenp*/Incenp gene knockout mice is phenotypically not distinguishable by eye from wild-type mice. And both in plants and in mice homozygous *wyr*/*wyr* respectively *Incenp*/*Incenp* knockout offspring from the heterozygotes is not viable. Embryo lethality in mice homozygous *Incenp*/*Incenp* knock out offspring is mainly explained by chromosome missegregation leading to aneuploidy[3]. Chromosome missegregation due to failures in sister chromatid cohesion has been described in *Drosophila incenp* disrupted oocytes during meiosis 1 and 2[4].

Here we confirm the chromosome missegregation phenotype in heterozygous

wyr-1/WYR plants and find Sli15 in yeast to be asymmetrically distributed during cell divisions.

We observed an enrichment of DAPI stained DNA in arrested mononuclear pollen and the preferential segregation of the functional *WYR* allele into arrested mononuclear pollen from heterozygous *wyr-1*/*WYR* plants. Based on these findings we come up with a new model, which describes a new condition causing haploinsufficiency. A WYR protein gradient is built due to the reduced WYR expression in heterozygous *wyr-1*/*WYR* microsporocytes. Subsequently, this gradient leads to an accumulation of DNA at the pole with the functional *WYR* allele, which is more enriched for the protein. The accumulation of DNA at one pole leads to chromosome duplications at the corresponding pole and chromosome deletions at the opposite pole.

Results

The segregation of the wild-type *WYR* and mutant *wyr-1* allele is not random

Incenp in mouse[3] and *incenp* in fly[4] causes defects during female meiosis. To check whether phenotypes observed for *wyr-1* are caused due to defects in meiosis, we isolated single arrested mononuclear pollen from mature pollen, whole genome amplified the genomic DNA and genotyped for the wild-type *WYR* or mutant *wyr-1* allele by PCR and Sanger sequencing (Figure 1A, B and C). If the pollen were arrested due to defects during or just before pollen mitosis 1, we would expect a frequency of about 100% *wyr-1* allele segregation to this pollen. Otherwise, if the pollen were arrested due to defects during meiosis we would expect a frequency of about 50% *wyr-1* allele segregation to this pollen. We analyzed 14 arrested mononuclear pollen from a heterozygous *wyr-1*/*WYR* plant. Due to the loss of the allele region or fragmentation of the allele region during single cell DNA isolation, we could only genotype 7 of the 14

isolated nuclei. Unexpectedly we genotyped for 6 pollen the wild-type *WYR* allele and for 1 pollen the *wyr-1* mutant allele (Figure 1D). Since most of the arrested pollen inherited the wild-type allele, the defect causing this phenotype must have occurred before pollen mitosis 1.

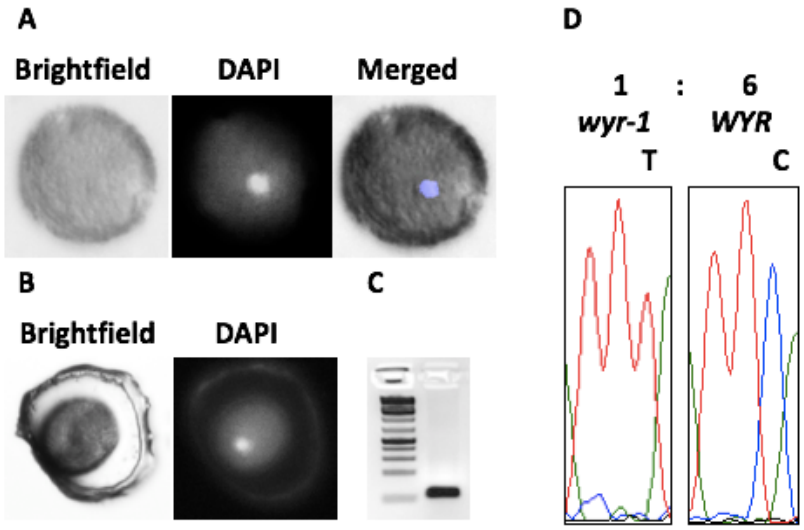
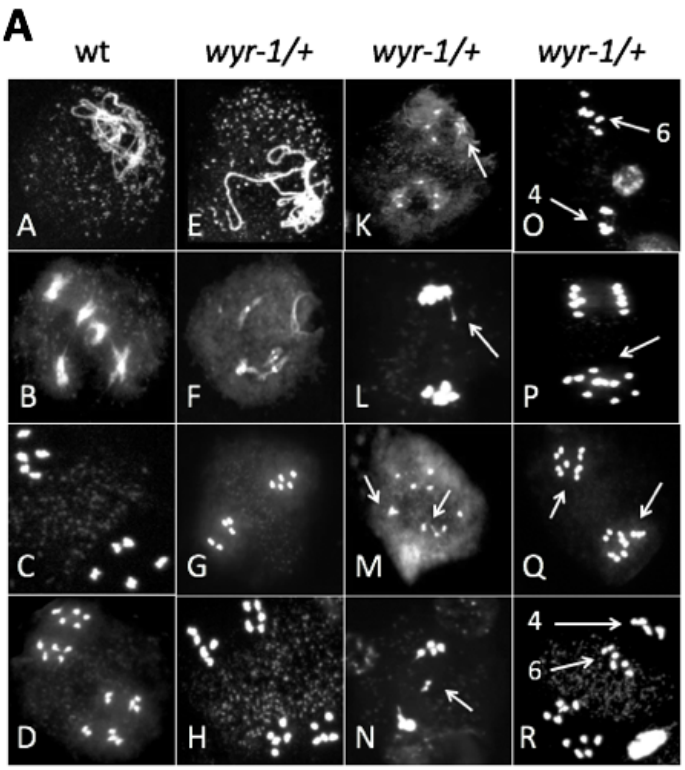


Figure 1 Isolation and genotyping of arrested mononuclear *wyr-1*/*WYR* pollen. **A)** Brightfield, DAPI and a merged picture of an arrested mononuclear pollen with only one nucleus. **B)** Laser capture microdissection isolated arrested mononuclear pollen. **C)** PCR product to genotype for the *wyr-1* or *WYR* allele. **D)** Sanger sequencing results of 7 genotyped mononuclear pollen. The *wyr-1* allele bears a C>T nonsense mutation. For 6 pollen we genotyped the wild-type *WYR* allele and for one pollen the *wyr-1* mutant allele.

wyr-1 leads to chromosome missegregation during pollen meiosis

As in plants, heterozygous *Incenp*/Incenp gene knockout mice is phenotypically not distinguishable by eye from wild-type mice. And both in plants and in mice homozygous *wyr*/*wyr* respectively *Incenp*/*Incenp* knock out offspring from the heterozygotes is not viable. Embryo lethality in mice homozygous *Incenp*/*Incenp* knockout offspring is mainly explained by chromosome missegregation leading to aneuploidy[3]. Chromosome missegregation has also been described in *Drosophila incenp* disrupted oocytes during meiosis 1[4]. These results reported for the orthologs of *WYR* and the phenotype of 20% aborted, 15% arrested mononuclear and 1% arrested dinuclear pollen in heterozygous *wyr-1*/*WYR* plants[1] point to rather a chromosome segregation defect during pollen

meiosis than a defect in pollen mitosis. To assess chromosome missegregation during pollen meiosis, we prepared chromosome spreads[5] of microsporocytes from *wyr-1/WYR* heterozygous plants (Figure 2). Our results clearly demonstrate strong defects in meiotic chromosome segregation. We observed chromosome missegregation (Figure 2O and R), lagged chromosomes (Figure 2L – N), chromosome bridges (Figure 2L), cell-cycle synchronization problems (Figure 2P) and chromosome condensation defects (Figure 2K). None of these phenotypes has been observed in microsporocytes from wild-type plants. These results confirm *wyr-1* causing defects during plant meiosis. The chromosome segregation defects correspond to the described defects of *Incenp* in mouse and *incenp* in fly.



B

phenotype	*Ler* wt	*wyr-1/+*
normal	21	20
lagged chromosome	-	4
missegregation	-	2
unsynchronized	-	1
chromosome bridges	-	2
condensation problem	-	1

Figure 2 *wyr-1*: Chromosome missegregation during pollen meiosis. Chromosome spreads according to Armstrong *et al.* 2003[5] **A)** A-D) wild-type E-H) *wyr-1/WYR* A+E) pachytene stage B+F) diakinesis stage C+G) anaphase 1 D+H) anaphase 2 K-O) anaphase 1 P-R) anaphase 2 O+R) chromosome missegregation L-N) lagged chromosomes L) chromosome bridge P) cell cycle synchronization defect 2K) chromosome condensation defect **B)** quantitative data

Arrested mononuclear pollen from *wyr-1*/*WYR* heterozygous plants is enriched in DNA

We observed the preferential segregation of the functional *WYR* allele into arrested mononuclear pollen and chromosome segregation defects during pollen meiosis. To test whether arrested mononuclear pollen arises due to chromosome segregation defects during meiosis, we quantified DAPI stained DNA in both mature tricellular pollen and arrested mono- or dinuclear pollen. We observed a high enrichment of DNA in mononuclear and a slight enrichment of DNA in dinuclear pollen. The lesser enrichment of DNA in dinuclear pollen might explain why this pollen made it through pollen mitosis 1. Altogether this result points to chromosome duplications in arrested mononuclear pollen.

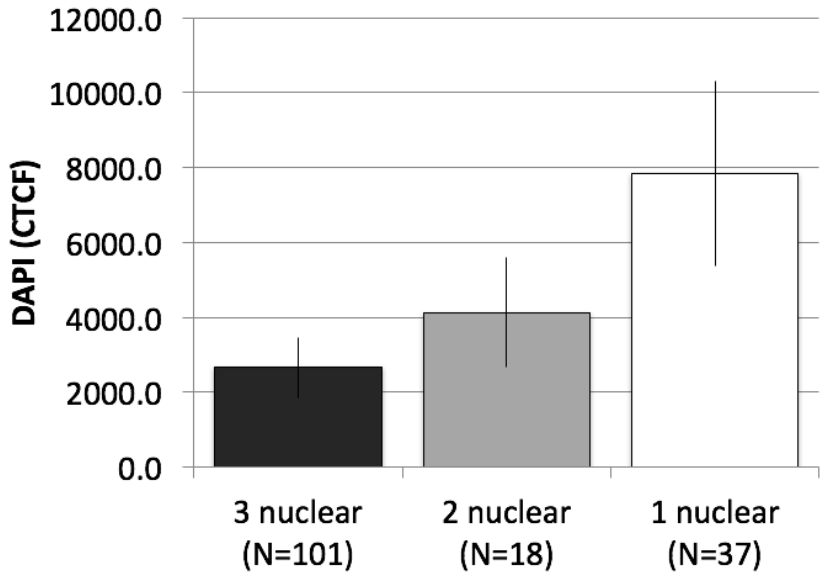


Figure 3 DNA quantification in mature 3-, 2- and 1 nuclear pollen. Mononuclear pollen have an increased amount of DNA.

Asymmetric GFP-Sli15 protein distribution during yeast mitosis

Sli15 is required for the asymmetric distribution of the kinetochore protein Ndc10 during mitosis[6]. Accordingly, we expected a similar distribution pattern for Sli15. To test this, we analyzed spores from a diploid strain bearing the two fluorescence tagged genes GFP-*SLI15* and *CNS1*-mCherry. *CNS1* is 170bp apart from *SLI15* coding on the opposite DNA strand and cosegregates with the locus of *SLI15*. The haploid spores always contain either the GFP-tagged *SLI15* or the mCherry-tagged *CNS1* gene and both expressed fusion proteins (Figure S1). By selecting spores having a stronger mCherry signal (Figure S2), we picked those spores, which inherited the mCherry-tagged *CNS1* gene but not the GFP-tagged *SLI15* gene. Nonetheless, these spores inherited a certain amount of nonencoded GFP-Sli15 protein that has already been expressed in the ancestor diploid cell. This allowed us to measure specifically the distribution of the inherited Sli15 protein and to exclude the measurement of newly expressed Sli15 protein. Consequently, we could exclude that we measured the differences in the expression level between mother and bud cells. For the first mitosis after sporulation, we observed a significant (P=0.0015, student's t-test) 2:1 enrichment of Sli15 in the mother cell compared to the daughter cell (Figure 4) The same result we found for mitosis 2 and 3 after sporulation (Figure 4). This distribution pattern correlates only in part with the one described for Ndc10[6]. For Sli15 we observed no specificity for the mother lineage. The distribution was asymmetric in all cell divisions. For spores, which inherited and encoded for GFP-*SLI15*, we did not see an asymmetric distribution of GFP-Sli15 during cell divisions (Figure 4). Therefore, the Sli15 protein gradient seems to be balanced by gene expression.

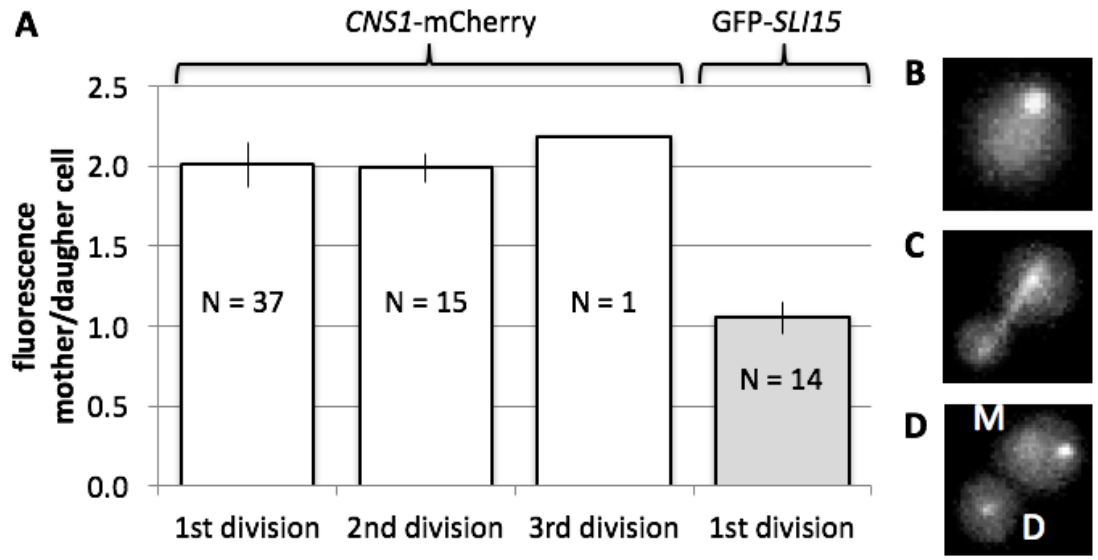


Figure 4 Asymmetric distribution of non-encoded GFP-Sli15 during mitosis. We measured and quantified the fluorescence of GFP-Sli15 after cell division when GFP-Sli15 relocated to the kinetochores (D). **A)** In spores coding for *CNS1*-mCherry and not for GFP-*SLI15*, we measured a 2-fold enrichment of the inherited protein during the first, second and third mitotic division of spores. We did not measure this enrichment in spores coding for GFP-*SLI15*. **B)** GFP-Sli15 localized to the kinetochores during prophase. **C)** GFP-Sli15 localized to the mitotic spindle during anaphase **D)** GFP-Sli15 relocalized to the kinetochores of both the mother and daughter cells after cell division.

Symmetric GFP-WYR protein distribution during plant male meiosis

The distribution of Sli15 protein is asymmetric in yeast. To check whether WYR is asymmetric in plants, we transformed plants with a *WYR*-GFP fusion gene. Transformed plants express the full-length mRNA but only partially complement the *wyr-1* phenotype when crossed to a plant heterozygous for *wyr-1*/*WYR* (Figure S3). To see the size of the protein, we enriched the translated WYR-GFP protein by immunoprecipitation against GFP and detected it with an anti-GFP antibody in Western blot analysis. The expected size of the protein based on bioinformatics calculations of the expected open reading frame is 196kDa. However, we only detected a specific band at 70kDa (Figure S4). To confirm the unexpectedly small band we let Eurogentec produce an antibody against an epitope within the conserved IN-box domain of WYR. Western blot analysis against WYR resulted in a band, of about 50kDa (Figure S5). Based on that results we suppose that

WYR is cleaved into a smaller protein of 50kDa. To check the symmetric or asymmetric distribution of WYR during meiosis, we did immunofluorescence chromosome spreads against WYR and analyzed the distribution of WYR during pollen meiosis metaphase 1. As a control, we performed immunofluorescence chromosome spreads against tubulin, which is expected to be symmetrically distributed. WYR is similar as tubulin symmetrically distributed at pollen meiosis metaphase 1 (Figure 5).

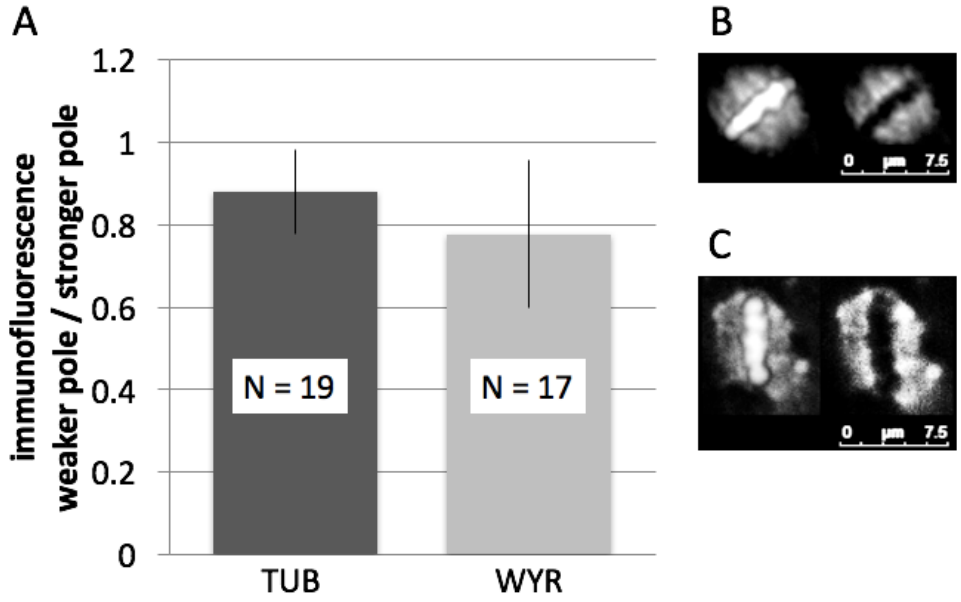


Figure 5 Immunofluorescence chromosome spreads against tubulin (TUB) and WYR. **A)** Immunofluorescence quantification against tubulin and WYR of chromosome spreads at pollen meiosis 1 metaphase. WYR is symmetrically distributed to both poles. **B)** Left: Immunofluorescence against tubulin and DAPI merged, Right: Immunofluorescence against tubulin **C)** Left: Immunofluorescence against WYR and DAPI merged, Right: Immunofluorescence against WYR

Discussion

wyr-1 causes chromosome segregation defects during pollen meiosis. Heterozygous *wyr-1*/*WYR* plants produce about 15% arrested mononuclear pollen from which 6 of 7 inherited the functional *WYR* allele and had an increased amount of DNA. 20% of pollen from heterozygous *wyr-1*/*WYR* plants abort. From these results, we suggest the working model (Figure 6) that arrested pollen asymmetrically inherits the functional *WYR* allele and thereby an increased risk for chromosome duplication, which in some cases leads to arrested mononuclear pollen. According to that working model, aborted mononuclear

pollen asymmetrically inherit the mutant *wyr-1* allele with a higher potential of chromosome loss, which always leads to pollen abortion. We suggest that the chromosome loss or chromosome duplication linked to the segregation of either the functional or mutated allele is caused by a WYR protein gradient, which leads to a lack of WYR protein at the pole receiving the mutant allele.

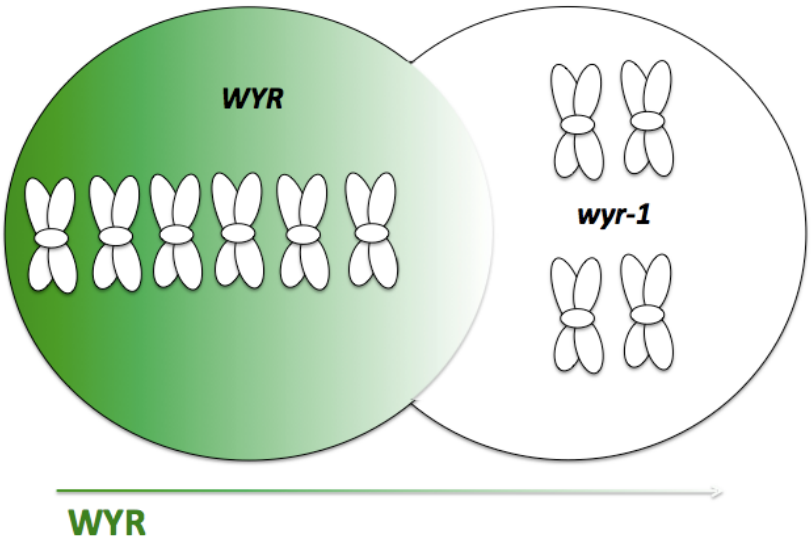


Figure 6 Working model. Heterozygous *wyr-1/WYR* plants build a protein gradient during pollen meiosis 1 due to a lack of *WYR* transcript. The protein level is increased at the pole with the functional *WYR* allele and decreased at the pole with the mutant allele. A lack of WYR protein enhances the risk for aneuploidy with the potential for missing chromosomes at the pole inheriting the mutant *wyr-1* allele and extra chromosomes at the pole inheriting the functional *WYR* allele.

Methods

Genotyping of arrested mononuclear pollen. Open flowers from RGS77 heterozygous *wyr-1/WYR* plants were cut and put into a tube containing water. After the addition of 2x DAPI solution (0.1M sodium phosphate, 1mM EDTA, 0.1% Triton-X-100, 0.4ug DAPI), the tube was vigorously vortexed. 1uL droplets of this pollen suspension were pipetted onto PE membrane-mounted metal frame slides and analyzed for the presence of mononuclear pollen (Leica DM6000). Pictures were taken to remember the position of the pollen. Slides were air dried at room temperature for about 1 hour and sprayed with

conventional hair spray (Migros I am Ultra Strong) to fix the pollen on the membrane. Slides were again air dried for about 1 hour. Microdissection was performed with an MMI CellCut Plus device (MMI Molecular Machines & Industries AG, Glattbrugg, Switzerland). Isolated pollen was collected using MMI isolation caps and stored at -80°C. All isolated samples were checked for the presence of the isolated pollen under an epifluorescence microscope.

Isolated pollen was directly lysed on the isolation caps by following the New England Biolabs PicoPLEX WGA Kit (New England Biolabs #E2620S) Pre-Amplification Protocol from step 1 to step 3. 5uL of cell extraction buffer and 5uL of extraction cocktail were pipetted into the isolation tube and by inverting the tube placed on the isolation cap. Parafilm wrapped caps were incubated in water baths according to step 3 of the protocol. After quick centrifugation, the PicoPlex WGA Kit Protocol was continued with step 4 of the PreAmplification protocol. Finally, the products of whole genome amplification were purified by a silica column (Macherey Nagel #740609.50 NucleoSpin Gel and PCR Clean-up kit). Before trashing the laser capture microdissection isolation caps, they were checked for the disappearance of any DAPI fluorescence to check for successful pollen lysis.

To genotype the *WYR* or *wyr-1* allele we amplified the allele region by PCR with primers RGS300 (TCCCAAAAGCAGAAGTTGCTA) and RGS301 (TGATGTTTCCTCTTGGAGTTATCT T) according to the Qiagen Multiplex PCR kit (Qiagen #206143) manual with a total reaction volume of 25uL, the annealing temperature of 60°C and 45 cycles. The PCR product was SANGER-sequenced with primer RGS300.

Chromosome spreads. Chromosome spreads were either done with flowers from heterozygous *wyr-1*/*WYR* RGS77 offspring plants (L*er* accession) or wild-type RGS32 offspring plants (L*er* accession). Inflorescences were fixed overnight in 3:1 ice cold ethanol : acetic acid. The fixative was replaced with 70% ethanol. All yellow anther-containing flowers were removed, and 70% ethanol was first replaced with citrate buffer pH4.5 and then with digestion medium (0.5% cellulase and 0.5% pectolyase in citrate buffer). After 10min incubation at 37°C, the digestion medium was removed and replaced with cold citrate buffer. Single flower buds were put on a glass microscope slide and covered with a cover slide. The buds were squeezed by tapping with a plastic pipette tip. After removing the cover slide the glass microscope slide was placed on a heat block at 45°C and immediately 10uL of 60% acetic acid were added. The slide was incubated for 1min at 45°C and air-dried. After the addition of Vectashield DAPI solution (VECTOR LABORATORIES, H-1200), the slides were analyzed under the epifluorescence microscope (Leica DM-6000, Leica, Wetzlar, Germany).

Chromosome spreads with immunofluorescence. Protocol adapted from Riechmann and Wellmer 2014[7]. Chromosome spreads were either done with flowers from heterozygous *wyr-1*/*WYR* RGS77 offspring plants (L*er* accession) or wild-type RGS32 offspring plants (L*er* accession). Inflorescences were fixed overnight in 3:1 ice cold ethanol:acetic acid. The fixative was replaced with 70% ethanol. All yellow anther-containing flowers were removed, and 70% ethanol was first replaced with citrate buffer pH4.5 and then with digestion medium (0.5% cellulase and 0.5% pectolyase in citrate buffer). After 10min incubation at 37°C, the digestion medium was removed and replaced with cold citrate buffer. Single flower buds were put on a glass microscope slide and covered with a cover slide. The buds were squeezed by tapping with a plastic pipette tip. And the slide was immediately put into a liquid nitrogen containing Dewar

vessel for a few minutes. Directly after removing the slide from liquid nitrogen the cover slide was removed and 50uL of the primary antibody (mouse α-TUB or guinea pig α-WYR) were added. After adding a new cover slide, the slide was incubated overnight at 4°C. After 3 wash steps in PBS, the secondary antibody was added (goat α-mouse(IgG)-Alexa488, ThermoFisher Scientific #A-11001 or Goat anti-Guinea Pig IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight 488 conjugate, Thermo Fisher Cat #: SA5-10094) and incubated for 2 hours at room temperature. After 3 washes in PBS, we added Vectashield DAPI solution and analyzed the slides under the epifluorescence microscope (Leica DM-6000, Leica, Wetzlar, Germany).

DAPI quantification in arrested mononuclear pollen. Open flowers from heterozygous wyr-1/WYR RGS77 plants were cut and put into a 1.5mL tube containing 100uL of DAPI solution (0.1M sodium phosphate, 1mM EDTA, 0.1% Triton-X-100, 0.4ug DAPI). The tube was vortexed, and 10uL of the solution were pipetted onto a glass microscopy slide. For each pollen grain, Z-stack data acquired on the Leica DM-6000 epifluorescence microscope (Leica, Wetzlar, Germany) equipped with an sCMOS camera (ANDOR, UK, Neo sCMOS DC152Q-FI) was compressed into a single plane using the SUM Slice function in ImageJ. Individual nuclei were selected using the freeform drawing tool to produce a region of interest (ROI). By using the 'Measure' function the area, the mean gray value and integrated density of the ROI were measured. The mean background was measured by repeating the same procedure for the non-fluorescent region around the nucleus. Finally, the corrected total nucleus fluorescence (CTNF) was calculated using the formula: CTNF = Integrated density of nucleus ROI – (Area of ROI x Mean fluorescence of background).

Yeast strain construction. We generated two transgenic yeast strains of the BY4742 ecotype: RGS11 (GFP-SLI15, mat-a) and RGS45 (CNS1-mCherry, mat-α).

GFP-Sli15 protein distribution quantification. To analyze the distribution of GFP-Sli15 between mother and daughter cells during mitosis without considering differences in gene expression, we followed the method from Thorpe *et al.* 2009[6]. The two haploid strains RGS11 and RGS45 were crossed, and diploid cells were isolated (RGS43 and 44). Diploid cells from RGS43 or RGS44 cell cultures were induced to sporulate by growing them in 1% potassium acetate. The sporulated culture was digested with Zymolyase to separate single spores from the tetrads and pipetted onto a round cover slide fixed in an attofluor cell chamber. On top of the cover slide, we added a SC agarose pad (SC-tryptophane medium with 1% agarose). The spores were grown for 2 hours at 30°C before they were put under the microscope. Microscopy was performed at 30°C. We programmed the microscope to take for each location on the sample every 15 minutes 20 pictures in the z-axis for 8 hours. Between 20 and 30 locations, each covering between 5-20 cells, were analyzed per run. For all spores classified as expressing C*NS1*-mCherry, we quantified the GFP-Sli15 fluorescence in both the mother and the bud cells by calculating CTNF (see DAPI quantification in arrested mononuclear pollen). We always quantified the signal at the first time point during anaphase when the signal localized to two spots (Figure 4D).

WYR-GFP **cloning** We cloned a construct consisting of 1.6kb endogenous *WYR* promoter, 8.0kb *WYR* genomic DNA and 0.7kb DNA coding for *GFP*. *WYR* promoter sequence was amplified with primer RGS146 (actagtggatcccccgggatgcatgCTTGCAGTTAT CTTACCCTCGGG) and primer RGS56 (CGCCGACGACCTACTCTAGAGATAGAGC). Primer RGS146 introduces a short tail (small letters) homologous to plasmid RGS27. WYR

genomic DNA was amplified with primer RGS130b (gctctatctctagagtaggtcgtcggcgATGTT TTCCGTCAAGGAGAATCCGAGG) and primer RGS131b (TCTCGACTGGAACTTTCGCGGC). Primer RGS130b introduces a short tail (small letters) homologous to the 3'-end of the *WYR* promoter sequence. DNA coding for GFP was amplified with primer RGS132b (gccg cgaaagttccagtcgagaGGATCCGTGAGCAAGGGCGAGG) and primer RGS133b (CGTTCA AACATTTGGCAATAAAgtttcttaagattgaatcctgttgc). Primer RGS132b introduces a short tail (small letters) homologous to the 3'-end of the *WYR* genomic DNA and Primer RGS133b introduces a short tail (small letters) homologous to plasmid RGS27. All 3 PCR products and linearized plasmid RGS27 were transformed into leucine auxotroph yeast to link the fragments by homologous recombination. Successful transformation circularizes the linearized plasmid bearing an *LEU2* gene. Through selection against leucine auxotroph cells, we isolated positive transformants. Plasmid isolated from positive yeast was transformed into *Agrobacterium tumefaciens.*

Plant transformation. *Arabidopsis thaliana* plants were transformed using a modified floral dip procedure[8,9]. Transformed progeny was selected by growing surface-sterilized T1 seeds on a Sucrose-free growth medium containing 50mg/L Hygromycin B (Sigma-Aldrich, Hygromycin B solution from Streptomyces hygroscopicus #H0654).

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Supplementary Information

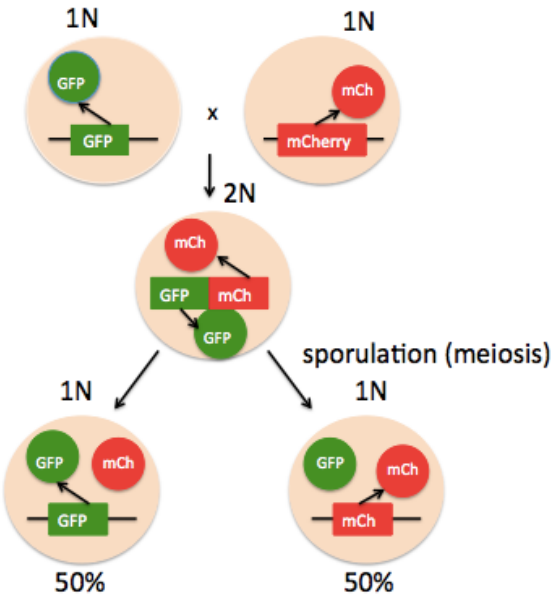


Figure S1 Protein distribution assay. The analysis of the distribution of a protein during mitosis requires a method, which can distinguish between differences in the gene expression level and real differences in the distribution of the protein. We crossed two haploid strains each bearing one of two tagged genes. Both are at the same genomic locus; one gene is tagged with GFP and the other with mCherry (mCh). The diploid resulting from the cross of those two strains possesses both tagged genes in the genome and also expresses both of them. Spores resulting from sporulation bear just either the GFP-tagged gene or the mCherry-tagged gene in their genome. However, both inherit both tagged proteins, which were expressed in the diploid strain before sporulation. The tagged protein encoded in the genome should be present in a higher amount because it is continuously transcribed. For example, to analyze the distribution of the GFP-tagged protein, we looked at spores, which expressed a stronger mCherry signal.

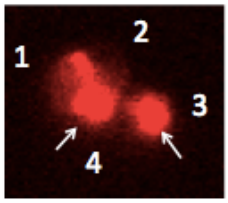


Figure S2 *CNS1*-mCherry expressed in tetrads. mCherry signal from a spore tetrad after sporulation of a diploid GFP-*SLI15*/*CNS1*-mCherry strain. *CNS1* encodes for a co-chaperone and locates to the cytosol. Spores 1 and 2 don't code for *CNS1*-mCherry whereas spores 3 and 4 code for *CNS1*-mCherry.

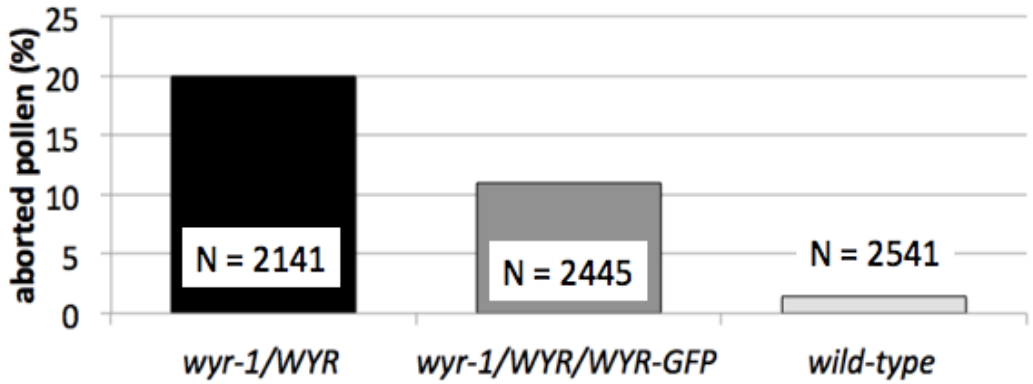


Figure S3 Alexander staining; functional complementation of *wyr-1* with *WYR*-GFP. Plants heterozygous for *wyr-1*/*WYR* have a pollen abortion phenotype with 20% pollen abortion.

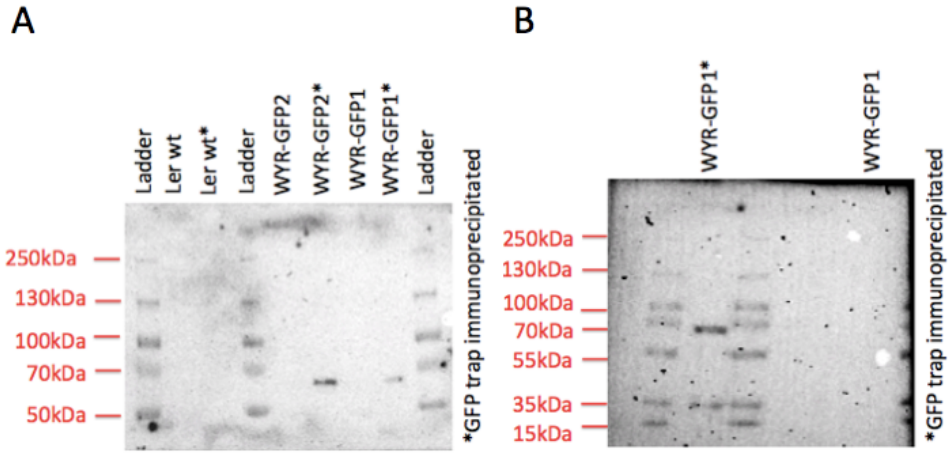


Figure S4 Western blot analysis against GFP. Proteins separated on a 7% SDS-PAGE gel were analyzed for the presence of GFP. **A)** Total protein from 100mg *wyr-1*/*WYR*/*WYR*-GFP seedlings, total protein from 100mg wild-type seedlings and GFP-immunoprecipitated protein from the same extracts was separated on an SDS-PAGE gel and transferred to a membrane. Immunodetection with an anti-GFP antibody resulted in a specific band of about 70kDa for the samples enriched for GFP proteins. **B)** Same experiment as in A) but with protein extracted from 300mg seedlings and separated on a 9% SDS-PAGE gel. GFP can't be detected in the raw extract but in the sample enriched for GFP. A second band at the size of free GFP is visible.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

My doctoral project started based on the hypothesis that *WYR*, a gene identified in a forward genetic screen for cell fate decision defects in the female gametophyte of *Arabidopsis thaliana*, is involved in the hypothetical non-random segregation of sister chromatids during gametophyte development.

To test this hypothesis, I developed an assay to track sister chromatid segregation during pollen development and further characterized *WYR*.

The assay I developed to track the segregation of the oldest DNA-strand containing chromatid during pollen development allows analyzing sister chromatid segregation in multicellular tissues. Furthermore, it allows analyzing sister chromatid segregation at a single chromosome resolution and to track rather the segregation of the oldest (synthesized the longest time ago) DNA-strand than the segregation of (+) and (-) strands. Tracking the oldest DNA strand, independent of its strandnes (+) or (-), is more suitable to test the immortal strand hypothesis.

It turned out that the oldest DNA-strand containing chromatids randomly cosegregate to either the vegetative or the generative nucleus during asymmetric pollen mitosis 1. Accordingly, directed sister chromatid segregation does not exist during pollen development.

This result rejects our hypothesis of *WYR* being involved in non-random sister segregation during gametophyte development.

Nevertheless, I observed the cosegregation of chromatids containing the oldest DNA-strand during pollen meiosis and pollen mitosis 1 in 50% of all analyzed pollen. All 5 chromatids containing the oldest DNA-strand segregated together into the same daughter cell during pollen mitosis 1 – either into the vegetative or generative cell.

Coordinated and directed sister chromatid segregation are the two essential conditions to be fulfilled to accept the immortal strand hypothesis. Accordingly, the existence of coordinated sister chromatid segregation during pollen development points to the existence of directed non-random sister chromatid segregation in other cell types, for example meristem cells, and supports the immortal strand hypothesis.

To test the immortal strand hypothesis one could look for the existence of coordinated and directed sister chromatid segregation in meristems. If coordinated sister chromatid segregation was present in meristems, the existence of directed sister chromatid segregation could be checked by just quantifying the segregation of EdU-FAM fluorescence.

My results give evidence to the existence of coordinated sister chromatid segregation but don't explain the mechanism responsible for it. Yadlapalli and Yamashita (2013)[1] published data pointing to the coordinated segregation of Watson or Crick strands (also referred as (+) and (-)) of autosomes, referred as WW::CC segregation, in diploid *Drosophila* germline stem cells (comment Sauer and Klar). Also, they found the directed segregation of mostly the same strand, (+) or (-), of both X and Y sex chromosomes to the same daughter cell during asymmetric germline stem cell division. They identified mutants of the LINC (linker of nucleoskeleton and cytoskeleton) complex, which showed random nondirected segregation of both sex chromosomes' sister chromatids. The LINC complex is composed of SUN- and KASH-domain proteins, which tether the nucleus to cytoskeletal components. Orthologs of the SUN domain proteins (AtSUN1 and AtSUN2)[2] exist in *Arabidopsis thaliana* and are therefore candidate genes to be involved in coordinated sister chromatid segregation during pollen development. By measuring and quantifying the segregation of EdU-FAM fluorescence during pollen mitosis 1 in *atsun1* and *atsun2* double mutant lines, one could check the candidate genes.

Alternatively, to find new genes involved in the coordinated non-random segregation of sister chromatids I suggest a forward genetic screen. In an automated assay, a collection of randomly mutagenized *Arabidopsis thaliana* plants could be quantified for the amount of EdU-FAM fluorescence segregation during pollen mitosis 1.

wyr-1 causes chromosome segregation defects during pollen meiosis. These defects can well explain the phenotypes described by Kirioukhova *et al.* 2009[3]. Furthermore, I observed an asymmetric distribution of Sli15, the yeast ortholog of WYR during mitosis and the preferential segregation of the functional *WYR* allele into arrested mononuclear pollen of heterozygous *wyr-1*/*WYR* plants. Based on these results, I suggested the working model that a gradient of WYR protein, caused by the lack of gene expression in *wyr-1*/*WYR* heterozygous plants, leads to a missegregation of chromosomes. However, to provide further evidence for that model it was important to test first the presence of a protein gradient in *Arabidopsis* and second the correlation of this gradient with the inheritance of the functional *WYR* or mutant *wyr-1* allele. Further my results point to the production of aneuploid gametes in *wyr-1* mutants. During my project work, I observed several *wyr-1*/*WYR* heterozygous plants with phenotypes like massively enlarged plants (extended inflorescences, large stem diameter, big seeds) or dwarf plants but I never quantified or further analyzed these effects. Also, I observed unexpected SNP ratios pointing to aneuploidy when I genotyped *wyr-1*/*WYR* heterozygous plants for the presence of the *wyr-1* causing SNP. For these reasons, I suggest checking *wyr-1*/*WYR* offspring plants for aneuploidy.

Since *WYR* is conserved from yeast to human and mutant alleles in plant, mouse, and fly have similar phenotypes, my results might also be useful to describe disorders in human. According to my working model, aneuploid offspring would not inherit the

mutant *wyr-1* allele. Hence, it would not be possible to identify *wyr-1* or its orthologs by a genetic screen in the aneuploid offspring. *wyr-1* could be only identified in the parents. However, in human studies, this data is often not available. Based on my working model about 5% (20% aborted pollen – 15% arrested mononuclear pollen) of all pollen may be assumed to be polyploid in *wyr-1/WYR* heterozygous plants.

References

1. Yadlapalli, S. & Yamashita, Y. M. Chromosome-specific nonrandom sister chromatid segregation during stem-cell division. *Nature* **498,** 251–4 (2013).
2. Graumann, K., Runions, J. & Evans, D. E. Characterization of SUN-domain proteins at the higher plant nuclear envelope. *Plant J.* **61,** 134–144 (2010).
3. Kirioukhova, O. *et al.* Female gametophytic cell specification and seed development require the function of the putative Arabidopsis INCENP ortholog WYRD. *Development* **138,** 3409–3420 (2011).
4. Loane, M. *et al.* Twenty-year trends in the prevalence of Down syndrome and other trisomies in Europe: impact of maternal age and prenatal screening. *Eur. J. Hum. Genet.* **21,** 27–33 (2013).

APPENDIX

Strand-specific single cell sequencing protocol

1.) EdU-FAM staining of pollen:

1.) Cut some young inflorescences and immediately put them into a 1.5mL Eppendorf tube filled with 100uM EdU (g). Put them through a hole (made with a needle / scissors) in the lead of the tube.
2.) Remove all open flowers and grow the inflorescences in a growth chamber for up to 9 days.
3.) Every day remove the anthers of open flowers, add about 50uL H_2O and destroy anthers with some needles to release the pollen. Pipette the released pollen into a PCR tube.
4.) Centrifuge at maximum speed for 2min. Remove the liquid but leave about 5-10uL.
5.) Mix 10uL of 6mM $Cu(II)SO_4$ with 0.3uL FAM-azide (6-Carboxyfluorescein azide) (b) stock solution
6.) Pipette this solution to the wall on one side of the PCR tube.
7.) Add 10uL of 30mM Sodium-L-ascorbate (a) to the opposite wall of the tube (to keep the solutions separated)
8.) Spin down to mix everything and vortex quickly.
9.) Incubate for 30min in the dark at RT.
10.) Centrifuge for 2min at max speed.
11.) Washing step1: Add 120uL of H_2O and vortex, centrifuge for 2min at max speed remove upper liquid but keep about 10uL in the tube.
12.) Washing step2: Repeat step 11.)
13.) Add 100uL of H_2O and store at -20°C

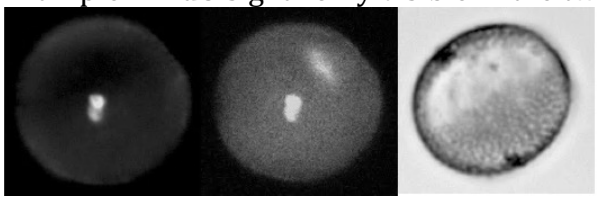
Material:

a) 30mM (+)-**Sodium-L-ascorbate** (Sigma Aldrich A7631): 297mg/50mL
b) 0.1M **6-Carboxyfluorescein-TEG azide** (Berry&Associates FF6110) stock: 5.7mg/100uL DMSO
d) 6mM **Copper(II)sulfate**: 47.8mg/50mL
g) 100uM EdU: 5mg EdU (Invitrogen) in 200mL MS + 2% Sucrose, store in 15mL falcon tube aliquots at -20°C
k) DAPI solution: 0.1M sodium phosphate (1mL of 1M stock)
1mM EDTA (20uL of 0.5M stock)
0.1% Triton-X-100 (10uL)
0.4ug DAPI/mL (2uL of 2mg/mL stock)

Example pictures of labeled Pollen:

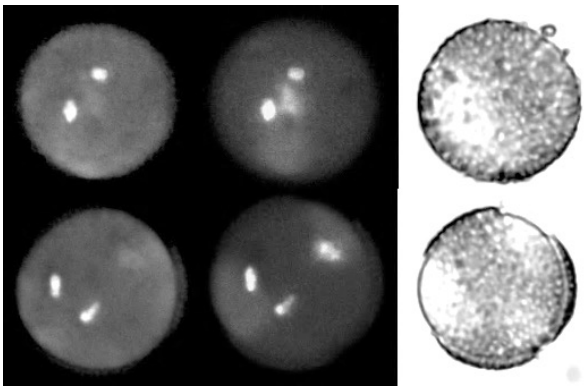
EdU (left), DAPI (middle), Brightfield (right)

Example 1: EdU signal only visible in the two generative nuclei



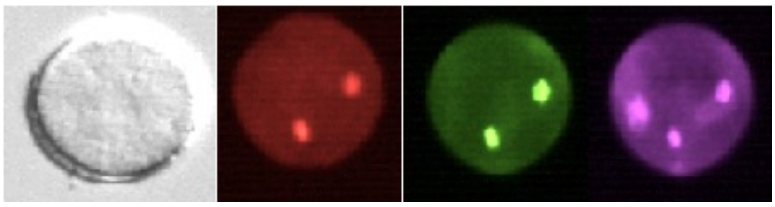
Example 2: Weak EdU signal visible also in the vegetative nucleus

Example 3 and 4: stronger EdU (but still weaker than in the generative) signal visible in the vegetative nucleus



Plant line used for the experiment: **RGS51**: Duo1:H2B-mRFP (generative cell specific mRFP expression):

Brightfield (gray), H2B-mRFP (red), EdU-FAM (green), DAPI (violet)



2.) Pollen lysis and laser capture microdissection

This protocol is designed to lyse pollen without separating the three nuclei. In the experiment, I used pollen from inflorescences grown in 100uM EdU and stained with 6-Carboxyfluorescine TEG-azide. The pollen was stored in 100uL H_2O at -20°C.

1.) Thaw the pollen and spin down at maximum speed for 2min.
2.) Remove the liquid (H_2O) but leave about 5-10uL in the tube.
3.) Add 100uL Sperm Extraction Buffer (SEB) and transfer the pollen into a 2mL (critical !) tube.
4.) Add 2 5-7mm glass beads (this size is critical !!!).
5.) Vortex for 30s at maximum speed.
6.) Add 10uL pollen DAPI solution.
7.) Remove the glass beads with a forceps.
8.) Pipette the liquid from the 2mL tube into a PCR tube.
9.) Spin down at maximum speed for 2min.
10.) Remove the liquid but leave about 5-10uL in the tube.
11.) Add 100uL H_2O, don't mix.
12.) Spin down at maximum speed for 2min.
13.) Remove the liquid but leave about 5-10uL in the tube.
14.) Add 100uL H_2O, don't mix.
15.) Put 1uL drops on a laser capture slide.
16.) Look for the fluorescence signal in the liquid drop (fluorescence not visible anymore as soon as the drop dried).
17.) Take pictures. One picture to recognize the position of the lysed pollen with Brightfield. Let the drops completely dry.
18.) Spray with hair spray so that the nuclei stick to the foil; hair spray: Migros I am Ultra Strong strength 5 (red bottle) 7613269346104
19.) Cut the nuclei at the laser microdissection microscope.

Material

Sperm Extraction Buffer (Borges *et al.* Plant Methods 2012):

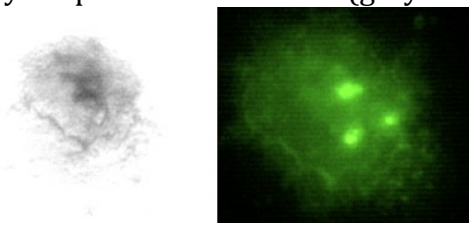
1.3mM H_3BO_3
3.6mM $CaCl_2$
0.74mM KH_2PO_4
438mM sucrose
5.83mM $MgSO_4$
7mM MOPS
pH 6.0

DAPI solution (10mL):

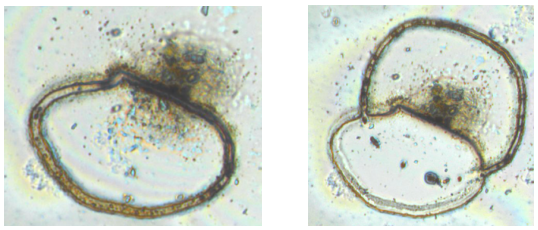
0.1M sodium phosphate (1mL of 1M stock)
1mM EDTA (20uL of 0.5M stock)
0.1% Triton-X-100 (10uL)
0.4ug DAPI/mL (2uL of 2mg/mL stock)

Example:

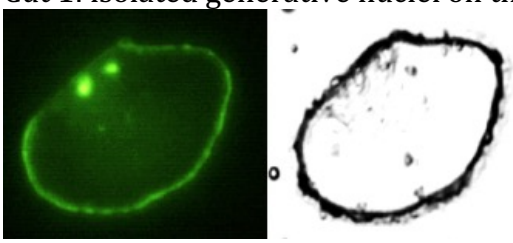
Lysed pollen before LCM: (grey: Brightfield ; green: EdU-FAM)



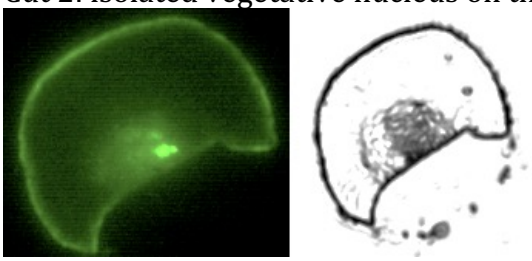
Cut 1 and 2 at the LCM



Cut 1: isolated generative nuclei on the lid of the LCM cap:



Cut 2: isolated vegetative nucleus on the lid of the LCM cap:



3.) Nucleus lysis, EdU-specific DNA digestion and whole genome amplification

Whole genome amplification of single cells labeled with EdU:

Overview:

1.) Cell/Nuclei lysis.
2.) Produce abasic sites at EdU positions with MUG uracil N-Glycosidase.
3.) Digest strands containing abasic sites with Exonuclease III.
4.) Whole genome amplification of strands containing no EdU with NEB PicoPlex WGA kit.
5.) Purification
6.) Quantification

Protocol:

1.)

a) Prepare a master mix:
   - 5uL cell extraction buffer
   - 5uL extraction cocktail

b) Remove the lid from the LCM tube and put it onto an Eppendorf 0.5mL lowbind tube, before pipette 10uL of the MM from step 1.)a) into the tube

c) Invert the tube and "shake" down the liquid so that all liquid is on the lid of the tube. Incubate inverted (on the lid) in a water bath (wrap the lid with parafilm !):
   - 10min @ 75°C
   - 4min @ 95°C
   - on ice

d) Spin down in a bench-top centrifuge.

2.)

a) add 1uL of MUG enzyme and incubate 10min @ 37°C.

3.)

a) add 1uL of Exonuclease III and incubate 5min @ 37°C

b) heat inactivation for 10min @ 70°C

4.)

a) add 5uL of Pre-Amp Cocktail (freshly prepared MM).

b) Incubate in old (black) thermocycler:
   - 2min 5s @ 95°C
   12 cycles:
   - 20s 95°C
   - 55s 15°C
   - 45s 25°C
   - 35s 35°C
   - 45s 65°C
   - 45s 75°C
   forever:
   - 4°C

c) centrifuge and pipette into a PCR tube

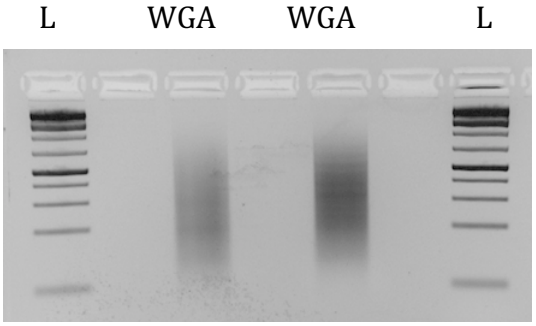
d) add 60uL of freshly prepared amplification cocktail to the 15uL preamp incubation product (step 4c) and mix gently by pipetting

e) amplify sample according to the thermal cycler program below:
   - 2min 95°C
   16 cycles:
   - 15s 95°C
   - 1min 65°C
   - 1min 75°C

5.) Column purification. Take care to choose the correct elution buffer.

6.) Quantify with nanodrop and run on Qiaxel; total expected yeald: 2-5ug/75uL = 27-67ng/uL

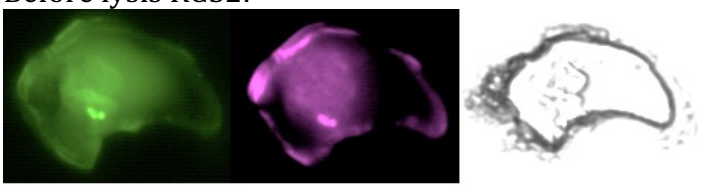
WGA products separated on a agarose gel:



Cell / nucleus lysis control:

To check, whether nuclei have been lysed I put the LCM lids back into the freezer and analyzed some of them again under the microscope:

RGS1 and RGS2: Pollen 9.6c1
Before lysis RGS2:



After lysis RGS2:

4.) NGS library preparation (NUGEN Ovation Ultralow)

For Illumina sequencing, I used the WGA products without further fragmentation (size is already between 200 – 1000bp) as DNA input material for the Nugen Ovation Ultralow library kit. I followed the kit's manual with slight modifications (e.g. bead purification protocol).

Protocol:

Strand-Seq: Library Construction protocol for single cell samples after whole genome amplification.

End Repair and Ligation:

Before starting:
- Thaw BLUE ER1 Ver 3; BLUE ER2 Ver 4; BLUE ER3
- Thaw the adaptor plate: spin for 5 to 10 minutes at RT at 1000Xg (to minimize cross contamination)

1.) Thaw the frozen samples and add 5uL of End Repair Master Mix to 10uL of template DNA (1-100ng
DNA) and mix (vortexing + centrifugation).
End Repair Master Mix:
3.5uL End Repair Buffer Mix (BLUE ER1 Ver 3)
0.5uL End Repair Enzyme Mix (BLUE: ER2 VER 4)
1.0uL End Repair Enhancer (BLUE: ER3)
2.) Place the tube in a pre-warmed thermal cycler programmed to run Program 1.
Program 1:
25°C, 30min (no heated lid)
70°C, 10min
4°C, forever
3.) Thaw the adaptor plate: spin for 5 to 10 minutes at RT at 1000Xg (to minimize cross contamination)
4.) Thaw YELLOW L1 Ver 4; YELLOW L3 Ver 4; GREEN D1
5.) Remove the tube from the thermal cycler and spin down.
6.) Unseal the needed wells of the adaptor plate.
7.) Add 12uL Ligation Master Mix into the appropriate well of the adaptor plate and mix.
8.) Immediately transfer the entire volume (15uL) from the adaptor plate well into the sample tube containing 15uL sample and mix.
Ligation Master Mix:
6.0uL Ligation Buffer Mix (YELLOW: L1 Ver 4)
1.5uL Ligation Enzyme Mix (YELLOW: L3 Ver 4)
4.5uL Water (GREEN: D1)
9.) Place the tube in a pre-warmed thermal cycler programmed to run Program 2.
Program 2:
25°C, 30min (no heated lid)
70°C, 10min
4°C, forever
10.) Remove the plate from the thermal cycler and spin down.

11.) Add 15uL nuclease free water
12.) Add 81uL of Magnetic beads (Agencourt AMPure XP). Beads should be at RT and shake the bottle to resuspend the beads before use.
13.) Mix thoroughly by pipette mixing 10 times. Let the mixed sample incubate for 5min at RT.
14.) Place the reaction onto a Magnet rack for 2min to separate the beads from the solution.
15.) Aspirate the cleared solution from the reaction plate and discard.
16.) Add 200uL of freshly prepared 70% EtOH to each well of the reaction plate and incubate for 30s at RT.
17.) Aspirate out the ethanol and discard.
18.) Dry for 5min at RT.
19.) Remove tube from the magnet rack and add 40uL elution buffer (Nuclease-free Water green: D1) and pipette mix 10 times. Let stand at RT for 3min.
20.) Place the tube onto the magnet rack for 3min.
21.) Transfer 36uL of the liquid into a 0.2mL PCR tube.

Amplification:

22.) Add 44uL Amplification Master Mix to each sample (total volume = 80uL) and mix.
Amplification Master Mix:
35uL AMP BUFFER MIX (RED: P1 Ver 2)
4.0uL AMP PRIMER MIX (RED: P2 Ver 5)
1.0uL AMPLIFICATION ENZYME MIX (RED: P3)
4.0uL DMSO
23.) Place the tube in a pre-warmed thermal cycler programmed to run Program 3.
Program 3:
a) 72°C, 2min
b) 94°C, 30s
c) 60°C, 30s
d) 72°C, 1min ; 18 cycles b-d
e) 72°C, 5min
f) 10°C, forever
24.) Remove the tube from the thermal cycler and spin down.
25.) Add 144uL of Magnetic beads (Agencourt AMPure XP). Beads should be at RT and shake the bottle to
resuspend the beads before use.
26.) Mix thoroughly by pipette mixing 10 times. Let the mixed sample incubate for 5min at RT.
27.) Place the reaction onto a Magnet rack for 2min to separate the beads from the solution.
28.) Aspirate the cleared solution from tube and discard.
29.) Add 200uL of freshly prepared 70% EtOH to each well of the reaction plate and incubate for 30s at RT.
30.) Aspirate out the ethanol and discard.
31.) Dry for 5min at RT.
32.) Remove tube from the magnet rack and add 40uL elution buffer (Nuclease-free Water green: D1) and pipette mix 10 times. Let stand at RT for 3min.
33.) Place the tube onto the magnet rack for 3min.
34.) Transfer 36uL of the liquid into a low binding tube.

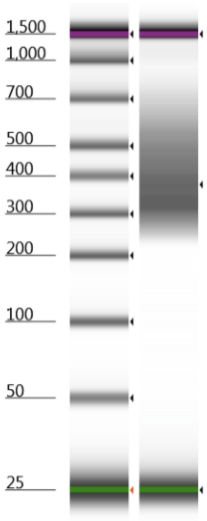
35.) Store Library at -80°C.

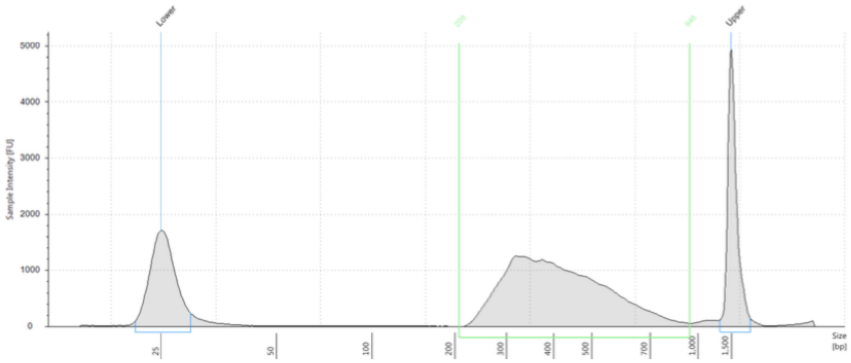
Material:

- 70% Ethanol ; SIGAM/FLUKA ; Cat No. 02877-1L ; Price: 42.30 Fr. for 1L
- Nugen Ovation Ultralow DR Multiplex System 1-96, Part No. 0329
- Beckman Coulter Agencourt AMPure XP magnetic beads

Finally, I measured the libraries at the FGCZ with Agilent Tape Station and pooled the libraries. For each library I pooled the same amount of DNA (amount depending on the measured DNA concentration at tapestation).

Final pooled and ready for sequencing library:





Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	% of Total	Region Comment	Color
209	946	431	18.8	72.6	93.61		

Table of yeast strains

Strain	Date	Creator	Description	Controls
RGS1	16.09.11	B	BY4742 mat-α; his3Δ; leu2Δ; met15Δ; ura3Δ	
RGS3	01.02.12	B	BY4741 mat-a; his3Δ; leu2Δ; met15Δ; ura3Δ	
RGS8	28.02.12	R	GFP-*SLI15* mat-α; transformed RGS1 colony e	PCR, Microscopy (no/weak signal)
RGS9	28.02.12	R	GFP-*SLI15* mat-α; transformed RGS1 colony d	PCR, Microscopy (no/weak signal)
RGS10	28.02.12	R	GFP-*SLI15* mat-a; transformed RGS3 colony o	PCR, Microscopy (strong signal)
RGS11	28.02.12	R	GFP-*SLI15* mat-a; transformed RGS1 colony n	CR, Microscopy (strong signal)
RGS12	28.02.12	R	GFP-*SLI15* mat-a; transformed RGS1 colony m	PCR, Microscopy (no/weak signal)
RGS23	28.02.12	R	GFP-*SLI15* mat-α; transformed RGS1 colony a	PCR, Microscopy (no/weak signal)
RGS24	28.02.12	R	GFP-*SLI15* mat-a; transformed RGS1 colony b	PCR, Microscopy (no/weak signal)
RGS25	28.02.12	R	GFP-*SLI15* mat-α; transformed RGS1 colony c	PCR, Microscopy (no/weak signal)
RGS26	28.02.12	R	GFP-*SLI15* mat-α; transformed RGS1 colony f	PCR, Microscopy (no/weak signal)
RGS27	28.02.12	R	GFP-*SLI15* mat-α; transformed RGS1 colony h	PCR, Microscopy (no/weak signal)
RGS50	15.10.12	R	GFP-*SLI15*; mat-a; Gal-HO plasmid; RGS11 transformed	PCR, Microscopy (strong signal)
RGS51	15.10.12	R	GFP-*SLI15*; mat-α; Gal-HO plasmid; RGS50 induced mating-type switch	PCR, Microscopy (weaker signal)
RGS45	10.08.12	R	*CNS1*-mCherry mat-α; transformed RGS1 colony 3	PCR, Microscopy (strong signal)
RGS46	10.08.12	R	*CNS1*-mCherry mat-α; transformed RGS1 colony 10	PCR, Microscopy (strong signal)
RGS43	24.08.12	R	RGS11xRGS45;GFP-*SLI15*;*CNS1*-mCherry	PCR, Microscopy (strong signal)
RGS44	24.08.12	R	RGS11xRGS45;GFP-*SLI15*;*CNS1*-mCherry	PCR, Microscopy (strong signal)

Table 1 Table of yeast strains. All yeast strains, which contributed to my results presented in this thesis. Creator describes who has produced the corresponding strain. R stands for me, B stands for the Yves Barral lab. For all strains, which I transformed I tested the correct genomic integration at the specific position in the genome by PCR.

Table of plant lines

seed number	genotype	ecotype	selection	date	description
RGS32	L*er* wild-type	*Ler*	-	17.09.12	-
RGS3/OK93	*wyr-1/WYR*	*Ler*	-	06.03.13	-
RGS28	*AtSUN1-GFP*	*Col*	Kan	06.08.13	from Yoshihisa Oda
RGS29	*AtSUN2-GFP*	*Col*	Kan	06.08.13	from Yoshihisa Oda
RGS30	*atsun1-1 AtSUN2-KD*	*Col*	Hyg	06.08.13	from Yoshihisa Oda
RGS51	*Duo1:H2B-mRFP*	*Col*	-	15.11.13	from David Twell
RGS77	*wyr-1/WYR*	*Ler*	-	12.01.15	-
RGS97	*WYR/WYR/WYR-GFP*	*Ler*	Hyg	26.01.16	T1_RGS32 transformed with Plasmid RGS26
RGS101	*wyr-1/WYR/WYR-GFP*	*Ler*	Hyg	04.02.16	T1_RGS77 transformed with Plasmid RGS26
RGS102	*RGS97 x RGS77*	*Ler*	Hyg	04.02.16	-
RGS104	*wyr-1/WYR/WYR-GFP*	*Ler*	Hyg	18.03.16	RGS102 selfed

Table 2 Table of plant lines. All plant lines, which contributed to my results presented in this thesis.

Table of plasmids

strain	plasmid description	stored in	bacteria selection
pRGS4	Gal-HO URA3 plasmid: to change yeast mating type by galactose induction	*E. coli*	Kanamycin
pRGS7	pRS315 described by Sikorki, R.S. and Hieter, P. 1989; *LEU2*; tested by restriction enzyme digestion: ok	*E. coli*	Ampicillin
pRGS6	pYM27; contains GFP-kanMX construct for C-terminal gene tagging; AmpR	*E.coli*	Ampicillin
pRGS21	pYB1137; contains mCherry-kanMX construct for C-terminal gene tagging; Neo	*E.coli*	Neomycin
pRGS3	pCB64 plasmid: contains GFP sequence used for plasmid pRGS26	*E.coli*	Kanamycin
pRGS27	p6U_yeast: T-plasmid for yeast cloning: yeast: LEU2 and ARSH4, E. coli/A tumefaciens: Spectinomycin, Plant: Hygromycin	*E. coli*	Spectinomycin
pRGS26	*WYRp-WYR-GFP* cloned into pRGS27	*A. tumefaciens*	Spectinomycin

Table 3 Taple of plasmids. All plasmids, which contributed to my results presented in this thesis.

Plasmid maps

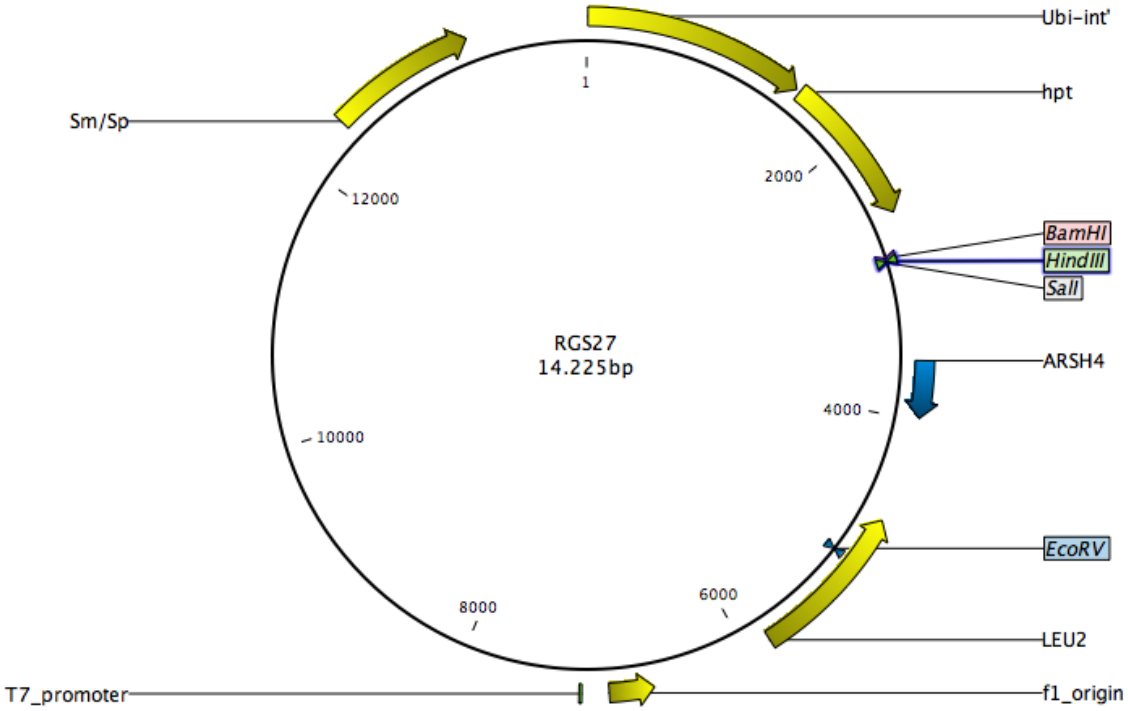


Figure 1 Plasmid RGS27. T-Plasmid to clone genes in yeast and transform into plants. Ubi-int: plant ubiquitin promoter, hpt: plant Hygromycin resistance gene, BamH1, HindIII, SalI: single cutter sites at the multiple cloning site, ARSH4: yeast origin of replication, LEU2: yeast LEU2 gene to select against leucine auxotroph yeast, f1_origin: bacteria origin of replication, Sm/Sp: Spectinomycin resistance gene to select against negative bacteria.

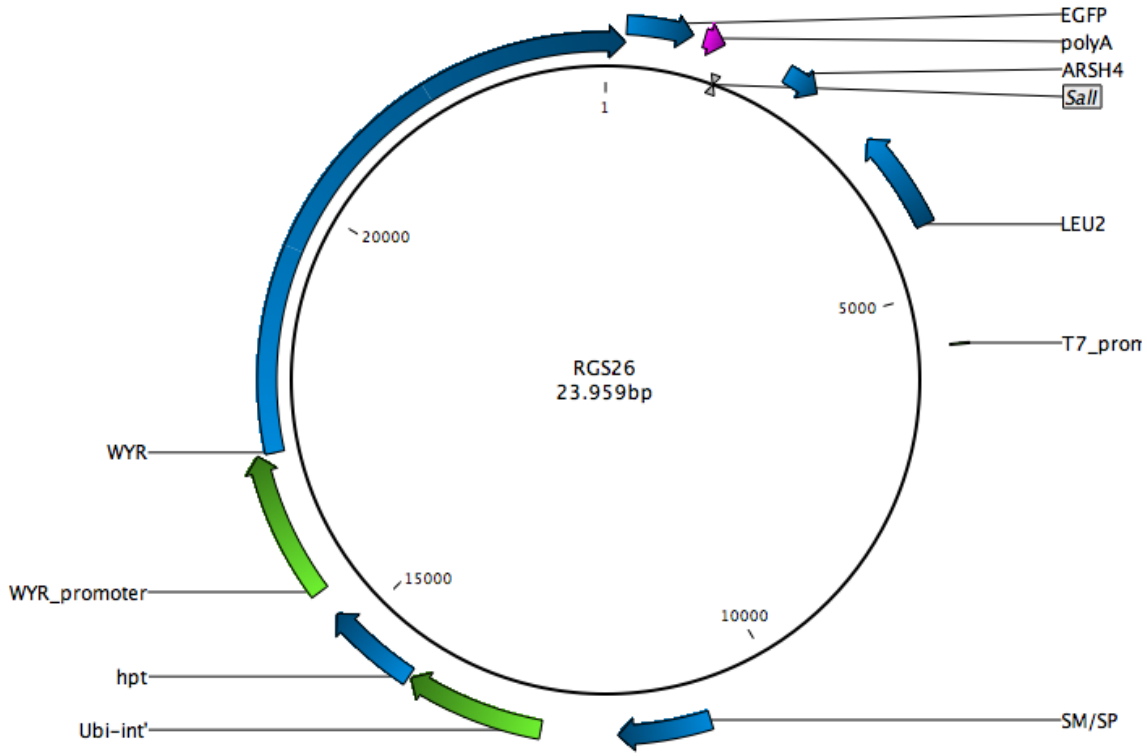


Figure 2 Plasmid RGS26. *WYRpromoter-WYR-GFP* cloned into RGS27 T plasmid. Ubi-int: plant ubiquitin promoter, hpt: plant Hygromycin resistance gene, SalI: single cutter site at the multiple cloning site, ARSH4: yeast origin of replication, LEU2: yeast LEU2 gene to select against leucine auxotroph yeast, f1_origin: bacteria origin of replication, Sm/Sp: spectinomycin resistance gene to select against negative bacteria. *WYRp*: 1.6kb genomic sequence upstream of *WYR* gene, *WYR*: genomic open reading frame of *WYR* gene without TAG stop codon, GFP: EGFP sequence from plasmid pRGS3.